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Delineating the role of *COX17* in the mitochondrial copper transport pathway

By

Fiona Ann Punter

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

In

Medical Sciences – Medical Genetics

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Dr. Bruce Hill

July 10, 2003
Date

ABSTRACT

Cytochrome oxidase, the terminal electron acceptor of the mitochondrial respiratory chain, is a multimeric enzyme complex located in the inner mitochondrial membrane. One important aspect of cytochrome oxidase biogenesis involves the incorporation of copper into the holoenzyme, where the heavy metal plays an essential role in electron transfer. Studies in the yeast, *Saccharomyces cerevisiae*, have led to the identification of Cox17p, a small, hydrophilic protein that is believed to function as a copper chaperone, shuttling copper from the cytosol to the intermembrane space of the mitochondrion for insertion into cytochrome oxidase. Since the initial identification of *COX17* in yeast, homologous *COX17* genes have been identified in virtually all eucaryotic organisms. In this thesis, the genomic structure, expression, and chromosomal location of the human *COX17* gene have been elucidated. Human *COX17* contains three exons and encodes a ubiquitously expressed transcript that is approximately 450 basepairs. Although the *COX17* gene was previously mapped to chromosome 13q14-21, the results presented here suggest that a *COX17* pseudogene maps to this region. Mapping experiments show that the gene encoding the COX17 protein is located on the long arm of chromosome 3 (3q). In addition to the characterization of the human *COX17* gene, the subcellular distribution of the human COX17 protein is described. Although a previous study has shown that mammalian COX17 is not found in the mitochondrion, a mitochondrial localization is demonstrated for human COX17 using two different experimental methods. To gain further insight into the function of yeast Cox17p, a mutagenesis study was carried out. Because several of the *cox17* mutants display a specific loss of cytochrome oxidase subunit II, the results presented suggest that Cox17p functions in the provision of copper exclusively to subunit II, acting upstream of Sco1p, a mitochondrial inner membrane protein involved in mitochondrial copper recruitment. Taken together, these experiments provide a framework for delineating the molecular basis of a subset of inherited cytochrome oxidase deficiencies.

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LIST OF ABBREVIATIONS AND NOMENCLATURE

Abbreviations

adPEO	autosomal dominant progressive external ophthalmoplegia
ANT	adenine nucleotide translocator
BAC	bacterial artificial chromosome
BCS	bathocuproinedisulfonic acid
bp	base pair
CD	circular dichroism
cDNA	complementary DNA
COX	cytochrome oxidase
CPEO	chronic progressive external ophthalmoplegia
Cu	copper
EPR	electron paramagnetic resonance
EST	expressed sequence tag
ETC	electron transport chain
FA	Friedreich ataxia
FAD	flavin adenine dinucleotide
FALS	familial amyotrophic lateral sclerosis
Fe-S	iron-sulfur
FMN	flavin mononucleotide
FTIR	Fourier transform infrared
HSP	hereditary spastic paraplegia
kb	kilobase

kDa	kiloDalton
KSS	Kearns-Sayre syndrome
LHON	Lebers Hereditary Optic Neuropathy
MELAS	mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes
MERFF	myoclonic epilepsy with ragged red fibres
MILS	maternally-inherited Leigh syndrome
MNGIE	mitochondrial neurogastrointestinal encephalomyopathy
MPP	mitochondrial processing peptidase
mtDNA	mitochondrial DNA
NARP	neuropathy, ataxia, and retinitis pigmentosa
nDNA	nuclear DNA
NMR	nuclear magnetic resonance
OXPHOS	oxidative phosphorylation
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PET	petite
ROS	reactive oxygen species
RT-PCR	reverse transcriptase polymerase chain reaction
SOD	superoxide dismutase
TCA	tricarboxylic acid cycle
TIM	translocase of the inner membrane
TOM	translocase of the outer membrane
XLSA/A	X-linked sideroblastic anemia and ataxia

Nomenclature

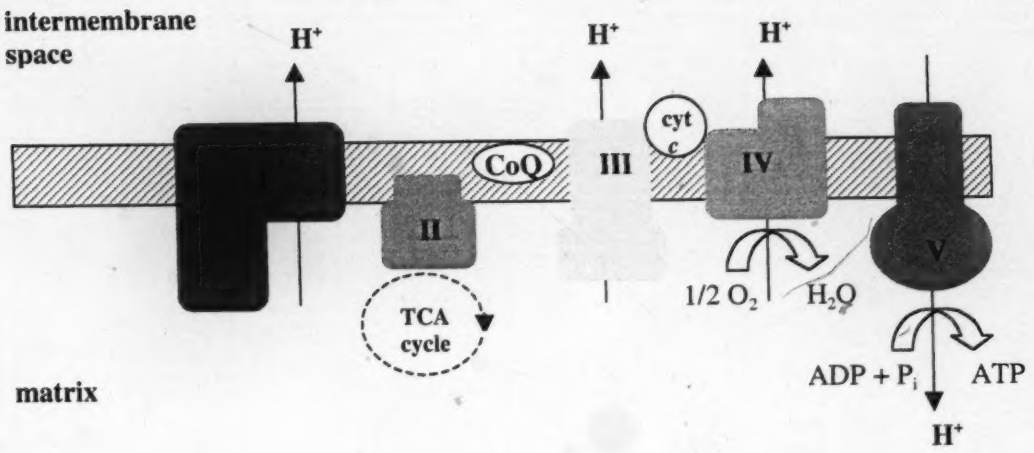
COX17	human gene
COX17P	human pseudogene
COX17	human protein
Cox17	mouse gene
Cox17	mouse protein
COX17	yeast gene
cox17	yeast mutant strain
Cox17p	yeast protein

Chapter 1. Introduction

OXIDATIVE PHOSPHORYLATION

Since the 1940's, the mitochondrion has been recognized as the major site of energy metabolism within the cell (Dahl and Thorburn, 2001). Through the process of oxidative phosphorylation (OXPHOS), a series of five multisubunit enzyme complexes embedded in the inner membrane of the mitochondrion generate ATP, the principal energy currency of the cell (Hatefi, 1985). The mitochondrial OXPHOS system (Figure 1-1) functions to couple the transfer of electrons to oxygen, which acts as the terminal electron acceptor in respiration, to the synthesis of ATP (Simon and Johns, 1999). As electrons are transferred through the first four complexes, comprising the electron transport chain (ETC), protons are concomitantly translocated from the mitochondrial matrix into the intermembrane space. The proton gradient thus established is then used to drive the synthesis of ATP from ADP and inorganic phosphate at complex V. The majority of ATP generated by OXPHOS is exported from the mitochondrial matrix by the adenine nucleotide translocator (ANT) for use in a diverse array of cellular processes (Smeitink et al., 2001).

The overall function of the first ETC enzyme, Complex I or NADH:ubiquinone oxidoreductase, is the oxidation of NADH and the transfer of electrons to the lipid-soluble electron carrier ubiquinone (van den Heuvel and Smeitink, 2001). Complex I is the largest of the ETC complexes and is comprised of at least 43 subunits. Seven of the subunits are encoded by mitochondrial DNA (mtDNA), while the remaining subunits are encoded by the nuclear genome. No high-resolution crystal structure is currently available for complex I, although low-resolution electron microscopic images indicate that the enzyme has an L-shaped structure (Yano, 2002). The hydrophilic portion of the



Name	Complex I NADH: ubiquinone oxidoreductase	Complex II Succinate: ubiqunone oxidoreductase	Complex III Ubiquinol: cytochrome *c* oxidoreductase	Complex IV Cytochrome oxidase	Complex V ATP synthase
Subunits	43	4	11	13	16
MtDNA Subunits	7 (ND1-6, ND4L)	0	1 (Cytb)	3 (COXI-III)	2 (ATPase 6, ATPase 8)
Redox cofactors	FMN, Fe-S clusters (8-9)	FAD, Fe-S clusters (3)	Cytochrome b_{566}, Cyto-chrome b_{562}, Cytochrome c_1, Fe-S cluster	Cu_A, Cu_B, Cytochrome *a*, Cytochrome a_3	-

Figure 1-1. Schematic of the mammalian mitochondrial OXPHOS complexes in the mitochondrial inner membrane. Electrons enter the mitochondrial respiratory chain (complexes I to IV) at complexes I (from NADH) or II (from the tricarboxylic acid [TCA] cycle). Electrons are transferred from complexes I and II to complex III by the electron carrier, ubiquinone (CoQ). Electrons are transferred from complex III to complex IV by the electron carrier, cytochrome *c* (cyt *c*). Molecular oxygen is reduced to water at complex IV. With the exception of complex II, all of the OXPHOS complexes translocate protons across the mitochondrial inner membrane (represented by a hatched bar). The proton gradient is used to synthesize ATP at complex V.

complex extends into the mitochondrial matrix and contains the majority of the redox cofactors involved in electron transfer, including flavin mononucleotide (FMN) and a number of iron-sulfur (Fe-S) clusters. The hydrophobic portion of complex I is embedded in the inner mitochondrial membrane and is thought to play a role in proton translocation (van den Heuvel and Smeitink, 2001). Along with complex IV deficiency, defects in complex I are the most frequently observed OXPHOS disorders in humans (Smeitink et al., 2001). Mutations have been identified in complex I subunit genes encoded by both the mitochondrial and nuclear genomes, with Leigh syndrome being the most common phenotype associated with complex I deficiency. Although no assembly factors for complex I have been identified to date, a study of complex I-deficient cell lines suggest that assembly defects do occur and that the proteins involved in this aspect of complex I biogenesis await identification (Triepels et al., 2001).

Complex II, or succinate:ubiquinone oxidoreductase, is the smallest of the ETC complexes and is involved in aerobic metabolism as part of the tricarboxylic acid cycle and the mitochondrial respiratory chain (Lancaster, 2002). The catalytic function of complex II involves the oxidation of succinate to fumarate and the transfer of electrons to the ubiquinone pool of the ETC. The transport of electrons through complex II occurs in the absence of proton translocation and therefore, it does not contribute to the proton gradient (Scheffler, 1999). Complex II is the only ETC complex that is encoded entirely by nuclear DNA (nDNA) and is composed of four different subunits. The flavoprotein and iron-sulfur protein subunits extend into the mitochondrial matrix making up the succinate dehydrogenase portion of the complex, which contains an FAD moiety and three Fe-S clusters, respectively (Rustin and Rotig, 2002). The two remaining subunits

are anchored in the mitochondrial inner membrane and contain a *b*-type heme, although the role of this cytochrome remains unclear (Lemire and Oyedotun, 2002). Currently, there is no crystal structure available for eucaryotic complex II and no assembly factors have been identified (Rustin and Rotig, 2002). Deficiencies in complex II are rare, accounting for approximately 2% of human respiratory chain deficiencies (van den Heuvel and Smeitink, 2001). Mutations in the subunit encoded by *SDHA* result in Leigh syndrome or a late-onset neurodegenerative disorder. Mutations in the remaining three subunits (encoded by *SDHB*, *SDHC*, and *SDHD*) have been reported in cases of hereditary paragangliomas and pheochromocytomas, suggesting that these genes act as tumour suppressors (Ackrell, 2002).

The third complex of the ETC is known as ubiquinol:cytochrome *c* oxidoreductase or the bc_1 complex because of the two cytochromes it contains (Scheffler, 1999). Complex III catalyzes the transfer of electrons from reduced ubiquinone to the mobile electron carrier, cytochrome *c*, with the concurrent translocation of protons across the inner mitochondrial membrane (van den Heuvel and Smeitink, 2001). The process of electron transport and proton translocation is carried out via a mechanism known as the Q cycle, which involves a bifurcated electron pathway through the enzyme complex (Scheffler, 1999). Of the 11 subunits of complex III, 10 are encoded by the nuclear genome. The remaining subunit, cytochrome b, is encoded by mtDNA. The four redox prosthetic groups involved in electron transfer include two *b*-type cytochromes, cytochrome c_1, and an Fe-S cluster that is part of the Rieske Fe-S protein subunit (Yu et al., 1999). Complex III can be divided into three regions: the matrix region extends into the mitochondrial matrix, the transmembrane region contains the two *b*-type hemes and

quinone binding sites, and the intermembrane space region houses the functional domains of cytochrome c_1 and the Rieske Fe-S protein (Xia et al., 1997; Yu et al., 1999). To date, no mutations have been detected in the 10 subunits encoded by nDNA (Shoubridge, 2001c). Mutations in the cytochrome b subunit gene result in human complex III deficiency with variable clinical phenotypes. A recent report has identified mutations in *BCS1L*, which encodes the only known human complex III assembly factor, that lead to tubulopathy, encephalopathy, and liver failure (de Lonlay et al., 2001). Defects in BCS1L have also been identified in GRACILE syndrome (growth retardation, aminoaciduria, cholestasis, iron overload, lacticacidosis, and early death), in which patients of Finnish heritage were found to have complex III activity within the normal range. This suggests that BCS1L has a function in addition to complex III assembly, possibly playing a role in cellular iron metabolism (Visapaa et al., 2002).

Cytochrome oxidase (COX), the fourth and final complex of the ETC, accepts electrons from reduced cytochrome c and passes them to molecular oxygen, the terminal electron acceptor of the mitochondrial respiratory chain. Complex IV also contributes to the proton gradient used to synthesize ATP (Taanman, 1997). A more comprehensive description of COX will be given below.

The condensation of ATP from ADP and inorganic phosphate occurs at the fifth complex of the mitochondrial respiratory chain, known as ATP synthase or the F_1-F_0 ATPase. Complex V can be divided into two subcomplexes, F_1 and F_0, which are connected by a "stalk" that consists of subunits from both subcomplexes. The F_1 subcomplex extends into the mitochondrial matrix and contains three catalytic sites where ATP synthesis occurs (Abrahams et al., 1994). The F_0 portion of the enzyme is

embedded in the inner mitochondrial membrane and acts as a proton channel, allowing the flow of protons from the intermembrane space into the mitochondrial matrix (Capaldi and Aggeler, 2002). ATP synthesis occurs by way of a rotary catalytic mechanism such that protons moving across the inner mitochondrial membrane cause components of the F_0 subcomplex to rotate. The conformational changes originating in F_0 are conducted to F_1 through the stalk, which results in the conformational changes needed to synthesize ATP at the active sites of F_1 (Wallace, 2001b). Two of the 16 subunits of complex V are encoded by the mitochondrial genome, with the remainder encoded by nDNA. Currently, all mutations identified in complex V OXPHOS disorders have been found in the mitochondrial *ATP6* gene, which encodes the 'a' subunit of the F_0 subcomplex. Phenotypic consequences of defects in *ATP6* include neuropathy, ataxia, and retinitis pigmentosa (NARP) and maternally-inherited Leigh syndrome (MILS) (DiMauro, 2001b). Two complex V assembly factors, shown to be involved in F_1 biogenesis in yeast, are known to have human homologues (Wang et al., 2001).

CYTOCHROME OXIDASE

Cytochrome oxidase, the terminal oxidase of many respiratory chains, catalyzes the transfer of electrons from cytochrome *c* to molecular oxygen (Iwata et al., 1995). Cytochrome oxidase is a member of the heme/copper-containing terminal oxidase superfamily, which is characterized by a high sequence similarity within the largest subunit (subunit I) of the oxidase and a binuclear active site containing a high-spin heme (heme a_3) and a copper atom (Cu_B) (Abramson et al., 2001). Because of their central role in energy metabolism, cytochrome oxidases have been studied extensively using

biochemical, genetic, spectroscopic, and crystallographic methods (Michel et al., 1998). Important structural and functional information has been obtained from the crystal structures of cytochrome oxidase, which has been solved to 2.8 Å resolution in the soil bacterium, *Parracoccus denitrificans* (Iwata et al., 1995), and in bovine heart mitochondria (Tsukihara et al., 1996).

The bovine cytochrome oxidase is composed of two 204 kDa monomers that face one another in the membrane, with each monomer containing 13 different polypeptide subunits (Tsukihara et al., 1996). Located within the subunits, intrinsic components of the COX complex include two heme A moieties, two copper sites, one zinc, one magnesium, and possibly some phospholipids (Tsukihara et al., 1995). The three largest subunits (subunits I-III) are encoded by the mitochondrial genome and form the core of the enzyme (Figure 1-2). Subunit I, which consists of 12 transmembrane helices and is located primarily within the membrane, makes contact with both subunits II and III. Subunit I contains the heme a site, as well as the heme a_3-Cu_B binuclear site where oxygen is reduced to water. Both of the heme molecules are perpendicular to the plane of the membrane and are buried within the enzyme, approximately 13 Å from the intermembrane space side of the membrane. Consistent with being a member of the heme/copper-containing oxidase superfamily, heme a, heme a_3, and Cu_B are coordinated to two, one, and three histidine residues, respectively. Subunit II consists of two transmembrane helices and a large extramembrane domain that extends into the mitochondrial intermembrane space and contains the Cu_A site. The Cu_A site is a dinuclear copper centre in which the two copper atoms are bridged by two sulfur atoms of cysteine residues. Each copper atom has two additional ligands, forming a coordination structure

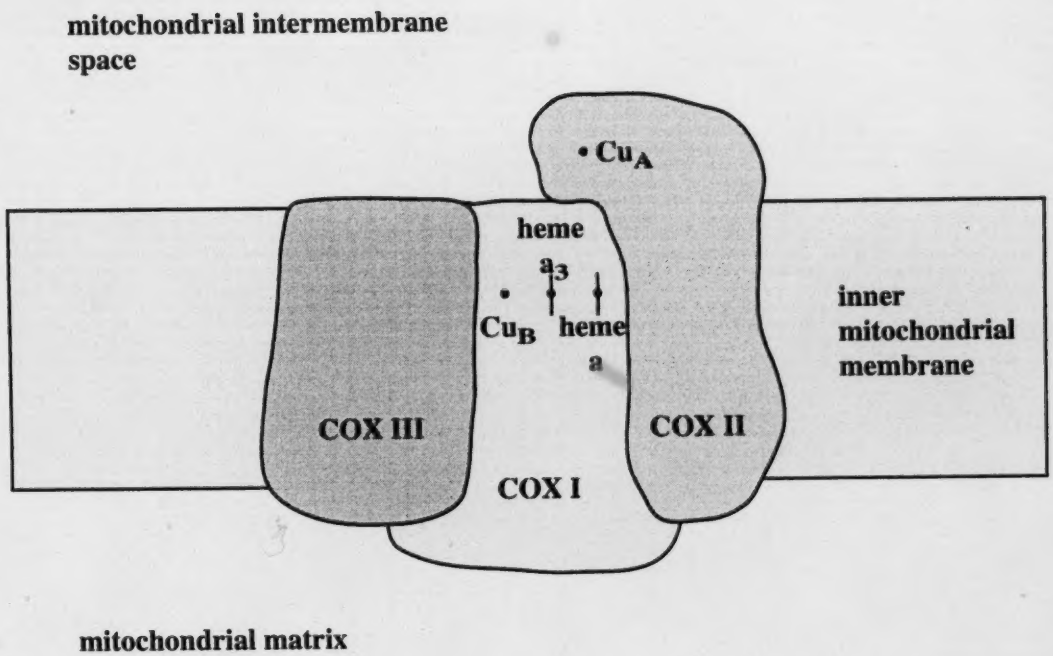


Figure 1-2. Schematic of the catalytic subunits of cytochrome oxidase. Subunit I (COX I), the largest subunit, contains 12 transmembrane helices, two *a*-type cytochromes and the Cu_B site. Subunit II (COX II), which contains two membrane-spanning regions, extends into the mitochondrial intermembrane space. The Cu_A site is found on subunit II. Subunit III (COX III) is primarily located within the inner mitochondrial membrane and contains seven transmembrane domains. Subunit I has an interface with both subunits II and III, although subunits II and III do not interact. The nuclear-encoded subunits form a shell around the exterior of the mitochondrial DNA-encoded core subunits. Electrons are transferred from the mobile electron carrier, cytochrome *c*, to the Cu_A site on subunit II, then to heme a on subunit I, and finally to the heme a_3-Cu_B binuclear centre on subunit I. The heme a_3-Cu_B binuclear centre is the active site of the enzyme, at which molecular oxygen is reduced to water.

that is similar to a 2Fe-2S cluster centre. This structure suggests that one electron is delocalized between the copper atoms when oxidized, such that one electron equivalent is sufficient for complete reduction during electron transfer (Yoshikawa, 1999). The seven α-helices of subunit III are contained mainly within the transmembrane region of the enzyme and are arranged to form a V-shaped cleft, which holds three phospholipids (Tsukihara et al., 1996). Although subunit III is a core component in the majority of known cytochrome oxidases, the subunit does not play a role in cofactor binding or proton translocation. While the function of subunit III remains unknown, it has been suggested that the subunit may be necessary for enzyme assembly or that it forms the entrance of an oxygen channel leading to the active site in subunit I (Michel et al., 1998). A non-redox active magnesium atom has been detected near the Cu_A site at the interface of subunits I and II (Tsukihara et al., 1995). The role of the magnesium atom remains to be defined, but it has been proposed to stabilize the Cu_A site (Tsukihara et al., 1995), strengthen the interaction between subunits I and II, control the direction of electron transfer, or facilitate proton transfer (Gennis and Ferguson-Miller, 1995).

In addition to the three core subunits, the crystal structure of bovine COX has confirmed the presence of ten subunits (subunits IV, Va, Vb, VIa, VIb, VIc, VIIa, VIIb, VIIc, VIII) encoded by the nuclear genome (Tsukihara et al., 1996). Seven of the ten nuclear-encoded subunits, each containing a single transmembrane α-helix, surround the three core subunits. The three extramembrane subunits are small globular proteins and include subunits Va, Vb, and VIb. Subunit Va, which is located below subunit 1, and Vb, which attaches tightly to the bottom surfaces of subunits I and III, are both found on the matrix side of the enzyme. Subunit Vb contains a zinc atom of unknown function,

arranged in a tetrahedral coordination by four cysteine ligands (Tsukihara et al., 1995). Subunit VIb associates with subunits II and III on the cytosolic side of the enzyme. The crystal structure shows that subunits VIa and VIb are involved in monomer-monomer interactions, suggesting that these two subunits play a role in stabilizing the dimeric state of the enzyme (Tsukihara et al., 1996). The function of the nuclear-encoded subunits remains unclear, particularly since the majority of the subunits are not found in the bacterial forms of the enzyme. The observation that the transmembrane helices of the subunits are arranged to provide the most stable helix-helix interactions indicates that the subunits may contribute to the conformational stability of the three core subunits (Yoshikawa et al., 1998). It has also been postulated that the nuclear-encoded subunits act as regulators, binding effectors such as nucleotides (Michel et al., 1998). The cholate binding sites identified in the crystal structure of the enzyme represent potential regulatory sites where effector molecules could bind (Tsukihara et al., 1996). The presence of tissue-specific isoforms of several subunits also points to a role in enzyme regulation (Michel et al., 1998).

Investigations into the kinetics of cytochrome oxidase have established that electron transfer occurs from cytochrome *c* to the heme a_3-Cu_B active site in subunit I via the Cu_A site in subunit II and the heme a in subunit I (Hill, 1991; Hill, 1994). The most effective mechanism for electron transfer through this hydrophobic environment is a hydrogen bond network involving side chains of amino acids, fixed water molecules, and other constituents (Yoshikawa et al., 1998). The crystal structure of bovine COX has suggested three possible hydrogen bond networks through the enzyme, but the actual path used in electron transfer has not yet been identified.

In addition to an electron transfer pathway, channels within COX are also required for molecular oxygen to reach the O_2 reduction site, for the removal of water from this site, and for the transfer of protons. Examination of the crystal structure has not led to the identification of an obvious oxygen channel. From studies of COX, it has been suggested that the enzyme contains two different types of proton channels, one for protons that are involved in the formation of water and one for protons that contribute to the proton gradient. Several hydrogen bond networks that may function to transfer protons have been identified in the crystal structure, but it is currently unclear which of these paths are used and whether conformational changes in the enzyme are involved (Yoshikawa, 1999). The exact nature of the mechanism that couples oxygen reduction with proton pumping remains unknown (Michel et al., 1998).

Deficiencies in complex IV, in addition to defects seen in complex I, are the most frequent cause of OXPHOS disorders in humans (Smeitink et al., 2001). Mutations causing COX deficiency have been detected in all three mtDNA-encoded subunits, resulting in a variety of phenotypes including myopathy, encephalomyophathy, sideroblastic anemia, and mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) (DiMauro, 2001b). While no mutations have been detected in the 10 COX subunits encoded by the nuclear genome, defects in five COX assembly factor genes have been identified in human COX deficiency. Human COX assembly factor genes will be discussed in greater detail below.

THE ASSEMBLY OF CYTOCHROME OXIDASE

While crystallography has provided detailed information about the holoenzyme structure of COX, very little is known in any organism about the mechanisms of holoenzyme assembly (Grivell et al., 1999). To date, COX biogenesis has been investigated to the greatest extent in the yeast, *Saccharomyces cerevisiae*, making yeast COX a popular model for the study of multimeric protein assembly in mitochondria (Poyton and McEwen, 1996). The discovery of genes involved in COX biogenesis has been largely due to the identification of yeast nuclear *petite* or *pet* mutants (McEwen et al., 1986; Tzagoloff and Dieckmann, 1990). *Pet* mutants are characterized by a lesion in a nuclear gene that renders the strain unable to use nonfermentable carbon sources (e.g. ethanol and/or glycerol), but able to grow on fermentable carbon sources such as glucose. When grown on medium containing glucose, the respiration-deficient *pet* mutants form smaller colonies (hence, the name "petite") than wild-type yeast. This altered morphology is due to the inability of *pet* mutants to metabolize the ethanol produced from glucose once glucose has been depleted in the growth medium (Tzagoloff and Dieckmann, 1990). While *pet* mutants can arise from defects in any one or several of the protein complexes involved in OXPHOS, a number of mutants with specific defects in COX have been identified (McEwen et al., 1986; Tzagoloff and Dieckmann, 1990). The majority of COX-deficient yeast strains lack spectrally detectable cytochromes a and a_3 (Tzagoloff and Dieckmann, 1990).

Initial studies characterizing 34 different COX-deficient *pet* mutant complementation groups in yeast suggested that many nuclear gene products are required for the expression of the COX subunits and for their subsequent assembly into the COX

holoenzyme (McEwen et al., 1986). Yeast COX is believed to be composed of 12 subunits (Taanman and Capaldi, 1992), with nine of the subunits encoded by the nuclear genome (Table 1-1). Analysis of *pet* mutants indicate that only certain nuclear DNA-encoded subunits are required for COX assembly, including subunits IV (*COX4*), Va (*COX5a*), VI (*COX6*), VII (*COX7*), and VIIa (*COX9*) (Poyton and McEwen, 1996). In addition to defects in nDNA-encoded COX subunits, mutations in *pet* genes can affect the processing and translation of mitochondrial COX subunit pre-mRNAs, heme A biosynthesis, mitochondrial copper import and insertion, and holoenzyme assembly (Table 1-2) (Poyton and McEwen, 1996). For many of the gene products involved in COX biogenesis, little is known about their function except that they play a role at some stage after the translation of the mtDNA-encoded subunits. For the majority of COX assembly genes that do have an assigned function, their exact mode of action remains to be delineated (Grivell et al., 1999). COX assembly mechanisms in yeast have proven difficult to study due to a lack of persistent COX assembly intermediates (Glerum and Tzagoloff, 1998; Grivell et al., 1999), as incorrectly or partially assembled COX complexes are recognized and degraded by the mitochondrial quality control system (Arlt et al., 1998) (Juhola et al., 2000).

PET Genes Causing a Pleiotropic Phenotype in Yeast When Mutated

While a number of *pet* genes have been shown to be specific for the biogenesis of a single OXPHOS complex, mutations in many additional *pet* genes compromise the function of multiple respiratory chain enzymes (Tzagoloff and Dieckmann, 1990). Not surprisingly, defects affecting several or all of the OXPHOS complexes are found in *pet*

Table 1-1. Yeast and bovine cytochrome oxidase subunit genes.

Yeast Gene	Genome	Yeast Subunit	Bovine Subunit
COX1	Mitochondrial	I	I
COX2	Mitochondrial	II	II
COX3	Mitochondrial	III	III
COX5a, COX5b	Nuclear	Va, Vb	IV
COX6	Nuclear	VI	Va
COX4	Nuclear	IV	Vb
COX13	Nuclear	VIa	VIa
COX12	Nuclear	VIb	VIb
COX9	Nuclear	VIIa	VIc
COX7	Nuclear	VII	VIIa
-	Nuclear	-	VIIb
COX8	Nuclear	VIII	VIIc
-	Nuclear	-	VIII

Table 1-2. Yeast nuclear *PET* genes exclusively involved in cytochrome oxidase assembly.

General Role	Gene	Function
Nuclear DNA-encoded subunit synthesis	COX4	Encodes subunit IV
	COX5a	Encodes subunit Va
	COX6	Encodes subunit VI
	COX7	Encodes subunit VII
	COX9	Encodes subunit VIIa
Assembly facilitator	*PET100*	Incorporation of subunit subcomplex (subunits VII, VIIa, VIII) into COX
Subunit I synthesis	*PET309*	*COX1* mRNA stability, translation
	PET54	*COX1* mRNA splicing
	MSS18	*COX1* mRNA splicing
	MSS51	Processing and translation of *COX1* mRNA
Subunit II synthesis	*MSS2*	Export of COX II C-terminal tail
	PET111	Translation of *COX2* mRNA
	COX18	Export of COX II C-terminal tail
	COX20	COX II maturation and assembly
Subunit III synthesis	*PET494*	Translation of *COX3* mRNA
	PET122	Translation of *COX3* mRNA
	PET54	Translation of *COX3* mRNA
Heme biosynthesis	*COX10*	Heme:farnesyl transferase (conversion of heme B to heme O)
	COX 15	Component of monooxygenase (conversion of heme O to heme A)
Copper Insertion	*COX17*	Putative mitochondrial copper chaperone
	SCO1	Copper provision to Cu_A site
	COX11	Copper provision to Cu_B site
Unknown	*PET117*	Unknown
	PET191	Unknown
	COX14	Unknown
	COX16	Unknown
	COX19	Unknown

genes that are involved in general processes of mitochondrial gene expression, including transcription, mRNA processing, and translation (Table 1-3).

The inner mitochondrial membrane contains a conserved proteolytic system composed of two AAA proteases, m-AAA (active site exposed to the matrix side of the inner membrane) and i-AAA (active site exposed to the intermembrane space side of the inner membrane). The AAA proteases, which mediate membrane protein degradation in eubacteria, mitochondria, and chloroplasts, contain an ATPase domain characteristic of the AAA superfamily (ATPases associated with diverse cellular activities) (Langer et al., 2001). Null mutations in any of the three genes encoding subunits of the AAA proteases lead to respiration defects, suggesting that in addition to their role in the turnover of unincorporated mitochondrial translation products, the AAA proteases are involved in the promotion of respiratory chain complex and ATP synthase assembly (Juhola et al., 2000).

Since OXPHOS proteins are encoded by both the mitochondrial and nuclear genomes, gene products destined for the inner mitochondrial membrane must be sorted from the location of synthesis prior to membrane insertion (Stuart, 2002). Mitochondrial proteins encoded by nuclear DNA are synthesized on cytosolic ribosomes and contain a mitochondrial targeting sequence, consisting of an N-terminal leader peptide or an internal sequence. The import of these proteins across the outer mitochondrial membrane is facilitated by the general import machinery, the translocase of the outer membrane (TOM) complex. Proteins traverse the inner mitochondrial membrane via the translocase of the inner membrane (TIM) complex. The Tim17-23 complex, which is used by precursor proteins containing an N-terminal mitochondrial targeting sequence, also involves Tim44p, the molecular chaperone mt-Hsp70p, and its co-chaperone, Mge1p.

Table 1-3. Yeast nuclear *PET* genes that cause a pleiotropic phenotype when mutated.

***PET* Gene**	**Function**	**Reference**
MAS1	Subunit of the mitochondrial processing protease	(Witte et al., 1988)
MAS2	Subunit of the mitochondrial processing protease	(Yang et al., 1988)
MIP1	Mitochondrial intermediate peptidase	(Isaya et al., 1994)
RPO41	Subunit of the mitochondrial RNA polymerase	(Kelly et al., 1986)
MTF1	Mitochondrial transcription factor	(Lisowsky and Michaelis, 1988)
MTF2/NAM1	Mitochondrial RNA processing and translation	(Asher et al., 1989)
PET112	Mitochondrial gene expression	(Mulero et al., 1994)
MRF1	Mitochondrial protein synthesis	(Pel et al., 1992)
MSS16	Splicing of *COB* and *COX1* premRNA	(Seraphin et al., 1989)
MRS1	Splicing of *COB* and *COX1* premRNA	(Bousquet et al., 1990)
PIM1	Matrix protease, processing and stability of *COB* and *COX1* premRNA, *COX1* translation	(Van Dyck et al., 1994; van Dyck et al., 1998)
MRS2	Component of mitochondrial Mg^{2+} influx system, splicing of group II introns	(Wiesenberger et al., 1992; Kolisek et al., 2003)
IMP1/PET2858	Subunit of inner membrane protease, protein processing and export	(Behrens et al., 1991; Schneider et al., 1991)
IMP2	Subunit of inner membrane protease, protein processing and export	(Nunnari et al., 1993; Schneider et al., 1994)
SOM1	Component of inner membrane protease, protein processing and export	(Esser et al., 1996; Jan et al., 2000)
AGF3/YTA10	Member of the AAA protease family	(Guelin et al., 1994; Paul and Tzagoloff, 1995)
RCA1/YTA12	Member of the AAA protease family	(Tzagoloff et al., 1994; Paul and Tzagoloff, 1995)
YME1/YTA11	Member of the AAA protease family	(Thorsness et al., 1993; Campbell et al., 1994)
OXA1/PET1402	Inner membrane protein insertion machinery	(Bauer et al., 1994; Bonnefoy et al., 1994; Hell et al., 2001)
MBA1	Component of mitochondrial protein export machinery	(Rep and Grivell, 1996; Preuss et al., 2001)

The Tim22-54 complex mediates the membrane insertion of some polytopic inner membrane proteins, including members of the metabolite carrier family. Once inside the mitochondrial matrix, the N-terminal mitochondrial targeting sequence of nDNA-encoded proteins is proteolytically processed by the mitochondrial processing peptidase (MPP). The export of both nDNA- and mtDNA-encoded proteins from the matrix, where mtDNA-encoded proteins are synthesized, into the inner membrane requires the highly conserved protein, Oxa1p. The inner membrane insertion of nDNA-encoded proteins is coupled with the export of their N-terminal regions to the intermembrane space, a process known as "N-terminal tail export". Since not all mtDNA-encoded proteins have their N-termini oriented to the intermembrane space, N-terminal tail export only occurs in some proteins encoded by the mitochondrial genome (for example, Cox2p). In addition to facilitating the export of N-terminal tails of some inner membrane proteins, Oxa1p has been shown to play a role in the insertion of polytopic inner membrane proteins whose N-termini are retained in the matrix, suggesting that it represents a component of general membrane insertion for mitochondrial proteins. Cross-linking studies have shown that Oxa1p interacts directly with nascent polypeptide chains being synthesized in the matrix, as well as imported nDNA-encoded proteins, as they are being exported from the matrix. Once inserted in the inner membrane, some proteins will undergo further proteolytic processing in the intermembrane space, carried out by the Imp1p/Imp2p protease (Stuart, 2002).

Oxa1p was first isolated from two independent genetic screens used to identify yeast proteins involved in respiratory chain complex biogenesis (Bauer et al., 1994; Bonnefoy et al., 1994). Oxa1p mutants were originally shown to lack COX activity,

although subsequent studies showed that the defects in *OXA1* also impaired complex III and complex V function (Stuart, 2002). In addition to the pleiotropic phenotype stemming from lesions in *OXA1*, mutations in other nuclear genes involved in various aspects of mitochondrial protein import, export, and processing also lead to defects in OXPHOS complex biogenesis. Mutations in either *MAS1* or *MAS2*, the genes encoding subunits of the mitochondrial processing peptidase, result in a *pet* phenotype, as do mutations in *MIP1*, which encodes the mitochondrial intermediate peptidase. Defects in Imp1p and Imp2p, which are components of the inner membrane protease, have been shown to impair respiration by preventing the processing and export of inner membrane proteins from the mitochondrial matrix (Behrens et al., 1991; Schneider et al., 1991; Nunnari et al., 1993; Schneider et al., 1994). Reduced assembly of several respiratory complexes in yeast with defects in *MBA1* have implicated its gene product in the insertion of some mitochondrially encoded inner membrane proteins (Rep and Grivell, 1996; Preuss et al., 2001). Although there is some overlap in the function and substrate specificity of Mba1p and Oxa1p, Mba1p may represent a mitochondrial inner membrane protein insertion pathway that is independent of Oxa1p (Stuart, 2002).

PET Genes Involved in Holoenzyme Assembly

While it is feasible that some mitochondrial proteins function in a chaperone-like capacity in COX assembly, it is currently unclear to what extent yeast *pet* genes are involved in the physical association of the COX subunits. However, recent evidence indicates that one protein, Pet100p (Church et al., 1996), may play a role in the incorporation of a subcomplex of subunits VII, VIIa, and VIII into the holoenzyme during COX assembly (Forsha et al., 2001).

***PET* Genes of Unknown Function**

A number of other yeast mutants have been described in which the characteristic features of a COX-deficient phenotype, such as reduced COX activity and the absence of the *a*-type cytochromes, have been observed. Studies of these strains have not detected any abnormality in the expression of the subunits encoded by either the mitochondrial or nuclear genome, nor is there an obvious aberration in heme A biosynthesis or copper insertion. This suggests that some other underlying defect is responsible for the compromised function or assembly of COX in these strains. *Pet* genes specific for COX assembly for which a function is unknown include *PET117*, *PET191* (McEwen et al., 1993), *COX14* (Glerum et al., 1995), *COX19* (Nobrega et al., 2002), and *COX16* (Carlson et al., 2003).

SHY1, a yeast *pet* gene of unknown function, encodes an inner mitochondrial membrane protein containing two putative transmembrane domains (Mashkevich et al., 1997). In the original study investigating Shy1p function, it was noted that steady-state levels of COX were reduced to 30% of wild-type levels in a Δ*shy1* strain. Since the assembled enzyme in the null mutant strain appeared to be structurally and enzymatically normal, it was suggested that Shy1p either facilitates COX assembly or increases the stability of the COX holoenzyme (Nijtmans et al., 2001). A recent report has identified Mss51p, a *COX1* mRNA-specific translation factor, as a multicopy suppressor of *shy1* mutants (Barrientos et al., 2002b). The suppression of COX defects in *shy1* mutants has led to the proposal that like Mss51p, Shy1p functions in some aspect of COX I metabolism (Barrientos et al., 2002b). Whether the reduction in COX assembly in *shy1*

mutants is due to the decreased presence of COX subunit 1, or to a more general defect in COX assembly, remains to be determined.

SHY1 (Surf Homologue of Yeast 1) was originally named for its homology to *SURF1*, which is a gene found in a cluster of housekeeping genes in mammals known as the surfeit locus (Zhu et al., 1998). While mutations in *SHY1* result in a pleiotropic phenotype in yeast, mutations in human *SURF1* are associated with isolated COX deficiency (Tiranti et al., 1998; Zhu et al., 1998), suggesting that the yeast and human proteins have different but overlapping functions (Zhu et al., 1998). Like Shy1p, SURF1 has a mitochondrial targeting sequence and has been localized to the mitochondrion (Zhu et al., 1998; Tiranti et al., 1999a; Yao and Shoubridge, 1999). Similarly to all of the human COX assembly factor genes identified to date, *SURF1* expression has been detected in all tissues tested (Yao and Shoubridge, 1999). Two-dimensional blue native polyacrylamide gel electrophoresis (PAGE) analysis of patient cells shows that mutations in *SURF1* cause an accumulation of COX intermediates, suggesting that *SURF1* mutants are blocked at an early step in COX assembly (Tiranti et al., 1999a). The majority of patients with defects in *SURF1* have a clinical phenotype of Leigh syndrome resulting from truncating mutations in the gene (Tiranti et al., 1998; Zhu et al., 1998; Tiranti et al., 1999b). *SURF1* is the first nuclear gene consistently associated with a relatively common OXPHOS disorder (Tiranti et al., 1999b). A single patient with villous atrophy, hypertrichosis, and mild neurological symptoms was found to be a compound heterozygote for a missense mutation and a 6 bp in-frame deletion in the *SURF1* gene (Von Kleist-Retzow et al., 2001). Although the patient displayed all of the typical molecular features found in individuals with Leigh syndrome, such as undetectable

SURF1, severe COX deficiency, and reduced levels of COX subunits, the absence of brain lesions in the patient indicates that defects in SURF1 do not exclusively lead to Leigh syndrome (Von Kleist-Retzow et al., 2001).

Heme A Biosynthesis

Heme A, found in both of the *a*-type cytochromes in COX subunit I, is unique to mitochondrial and some bacterial cytochrome oxidases (Tzagoloff et al., 1993). In the first step of heme A synthesis, the vinyl group on carbon 2 of the porphyrin ring of protoheme (heme B) is replaced by a hydroxyethyl farnesyl chain, yielding heme O. A second reaction results in the oxygenation or oxidation of the methyl group on carbon 8 of the porphyrin ring to a formyl group, yielding heme A (Svensson et al., 1993). A yeast *pet* mutant strain with a defect in the *COX10* gene (Nobrega et al., 1990) was found to be deficient in heme A, but not protoheme, suggesting that Cox10p is involved in one of the steps in the conversion of heme B to heme A. Studies in the bacteria, *Escherichia coli* and *Bacillus subtilis*, identified Cox10p homologues, CyoE and CtaB, respectively, which were shown to function as heme:farnesyl transferases (Mogi et al., 1994). These findings indicated that Cox10p, which restores heme A synthesis when transformed into a Δ*cox10* strain, may play the same role in yeast and is responsible for the farnesylation of the heme required by the COX holoenzyme (Tzagoloff et al., 1993).

The bacterium, *Rhodobacter sphaeroides*, contains a closely-related aa_3-type oxidase. Unlike *cox10* yeast mutants, which have severely reduced steady-state levels of COX subunit I, *R. sphaeroides* with mutated *cox10* shows subunit I at, or near, wild-type levels. Mutant analysis in the *R. sphaeroides* study suggested that heme A is not essential for expression, membrane insertion, or association of the COX core subunit

complex. Furthermore, it was proposed that heme A insertion may be able to occur at multiple steps in the assembly process and that assembly does not need to follow a linear or ordered pathway in *R. sphaeroides* (Hiser and Hosler, 2001). It is currently not clear what accounts for the difference in phenotype between the yeast and bacterial *cox10* mutants, although it is quite feasible that different assembly mechanisms exist for eucaryotic oxidases, which are located in the inner mitochondrial membrane, and bacterial oxidases, which are found in the plasma membrane.

A yeast strain carrying a mutation in the *COX15* gene (Glerum et al., 1997) was found to be deficient in heme A, despite containing heme O. This finding suggested that the *COX15* gene product is involved in the conversion of heme O to heme A (Barros et al., 2001). The oxidation of the methyl group to a formyl group at carbon 8 of the porphyrin ring in heme O is proposed to require an hydroxylation to the corresponding alcohol, which must be further oxidized to the aldehyde. The initial hydroxylation would be carried out by a monooxygenase, while the final step is a dehydrogenation. In the yeast, *Schizosaccharomyces pombe*, *COX15* is found to be fused to a homologue of the *S. cerevisiae* gene, *YAH1*, which encodes a mitochondrial ferredoxin, suggesting that in *S. cerevisiae*, Cox15p and Yah1p catalyze the hydroxylation or dehydration step in the conversion of heme O to heme A (Barros et al., 2001). Experimental evidence supports a role for both Cox15p and Yah1p in the formation of heme A in yeast (Barros et al., 2002). This pathway may also include a ferredoxin dehydrogenase, Arh1p, forming a three component monooxygenase that functions in heme A synthesis (Barros et al., 2001).

The human *COX10* gene, encoding a protein with high homology to yeast and bacterial farnesyl transferases, was isolated by functional complementation of a yeast *cox10* mutant (Glerum and Tzagoloff, 1994). Human *COX10*, which localizes to the short arm of chromosome 17 (17p), is ubiquitously expressed and contains no TATA or CAAT boxes, suggesting that *COX10* is a housekeeping gene (Murakami et al., 1997). When mutated in humans, patients show an isolated COX deficiency with a marked reduction in COX subunits III and VIc, and barely detectable levels of subunit II. Subunit I levels are relatively normal, despite an absence of heme A, suggesting that heme groups do not stabilize subunit I in human COX (Valnot et al., 2000b). Human *COX15* is a ubiquitously expressed gene found on chromosome 10q24 (Petruzzella et al., 1998). Defects in human *COX15* result in an isolated COX deficiency, with severely decreased COX activity in heart (Antonicka et al., 2003). Mitochondria isolated from an affected individual's heart muscle showed markedly reduced levels of heme A and increased levels of heme O. Blue-native PAGE analysis of patient heart tissue showed a decreased amount of assembled COX. While the patient still had detectable amounts of subunit I, subunits II, III, VIa, VIb, VIc, and VIIa were severely reduced in heart. Antonicka *et al.* (Antonicka et al., 2003) hypothesized that in the absence of COX15, the block in COX assembly occurs at S1, according to a human COX assembly pathway proposed by Nijtmans *et al.* (Nijtmans et al., 1998). Interestingly, despite involvement in the same biosynthetic pathway, mutations in *COX10* and *COX15* result in very different clinical phenotypes. Individuals with defects in *COX10* have been found to have leukodystrophy and tubulopathy (Valnot et al., 2000b), while mutations in *COX15* are manifested as fatal infantile hypertrophic cardiomyopathy (Antonicka et al., 2003).

COX Assembly in Human Cells

As in yeast cells, the basic mechanisms underlying COX assembly in higher eucaryotes, such as the order of subunit assembly and the regulation of assembly, remain to be resolved. Several preliminary studies have shown that in mammalian cells, COX assembly is a relatively slow and sequential process (Nijtmans et al., 1998). Using two-dimensional electrophoresis, Nijtmans *et al.* provided evidence for the existence of two assembly intermediates and a total of four species in the COX biosynthetic pathway in human cultured cells (Nijtmans et al., 1998). According to the COX assembly model proposed by Nijtmans *et al.* (Figure 1-3), the first species, S1, contains only COX subunit I. The second species, the S2 intermediate, consists of COX IV, in addition to COX I. This second intermediate seems feasible, given that a physical interaction between COX I and COX IV has been demonstrated in the COX crystal structure. The S3 intermediate was shown to contain all of the remaining COX subunits, with the exception of COX VIa and VIIa or b. S4, presumably representing the fully assembled COX holoenzyme, contained all of the COX subunits. The first three species identified in the assembly pathway may be detectable because they represent rate-limiting steps. S1, composed solely of COX I, may be the first rate-limiting step because of the addition of the bulky heme groups to subunit I before the association of the other subunits. The S2 species may be the second rate-limiting step because it involves the association of S2 with COXII, COXIII, and the majority of the nuclear encoded subunits. The fact that the S3 species is detectable as a subcomplex suggests that the incorporation of the two remaining nuclear subunits into the holoenzyme may be a relatively slow process. A

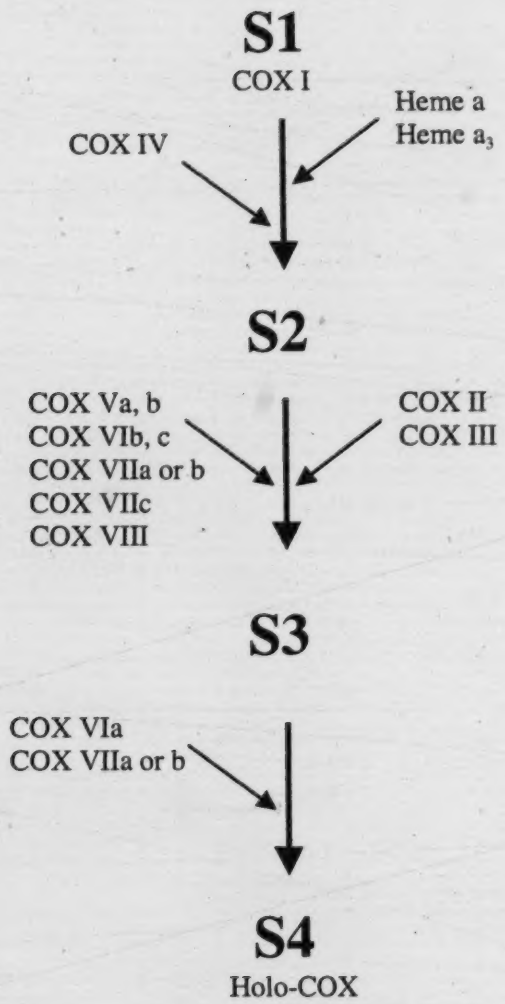


Figure 1- 3. Model for COX assembly in cultured human cells according to Nijtmans *et al.*, 1998. In the first subcomplex, S1, COX I is the only subunit present. Prior to the formation of S2, the heme groups are added to COX I, which associates with COX IV. In the third subcomplex, S3, the two remaining mtDNA-encoded subunits and the majority of the nDNA-encoded subunits assemble. In the last stage of assembly, COX VIa and COX VIIa or b are added to form the mature holoenzyme. Copper insertion is not depicted in this model. Adapted from Nijtmans et al, 1998.

similar dissection of the COX biosynthetic pathway remains to be carried out in yeast cells (Nijtmans et al., 1998).

COPPER IN CYTOCHROME OXIDASE ASSEMBLY

Cellular Copper

Copper (Cu) is an essential trace metal required for the normal growth, development, and function of all living organisms (Linder, 1991). Copper has been identified as a catalytic cofactor in enzymes involved in a variety of biological processes (Table 1-4), including respiration, iron transport, oxidative stress protection, peptide hormone synthesis, pigmentation, and blood clotting (Puig and Thiele, 2002). Deficiencies or defects in a number of these Cu-dependent enzymes have been shown to underlie disease states in humans or mice (Pena et al., 1999). Imbalances in copper, which stem from an inability to appropriately distribute copper to cells and tissues, also lead to serious conditions in humans, such as Menkes or Wilson disease (Puig and Thiele, 2002). In addition, copper has been suggested to play a pathophysiological role in several neurodegenerative diseases, including familial amyotropic lateral sclerosis (Waggoner et al., 1999), Alzheimer disease (Strausak et al., 2001), and prion diseases (Lehmann, 2002). Currently, the exact nature of copper involvement in these neurological conditions remains unclear (Mercer, 2001).

As a result of its ability to exist in both an oxidized [Cu(II)] and a reduced [Cu(I)] state, copper has been established as an essential redox cofactor in a number of Cu-containing enzymes (Labbe and Thiele, 1999). However, the redox properties of Cu also allow the heavy metal to readily participate in reactions that lead to formation of reactive

Table 1- 4. Mammalian copper-containing enzymes[a].

Protein	Biological Function	Consequence of Deficiency
Ceruloplasmin	Ferroxidase	Iron deficiency anemia, neurodegenerative disease
Clotting factor V, VIII	Blood clotting	Bleeding tendency[b]
Cu, Zn superoxide dismutase	Free radical detoxification	None without further manipulation[b]
Cytochrome oxidase	Terminal electron acceptor of the mitochondrial respiratory chain	Decreased ATP synthesis causing encephalopathy and/or myopathy
Dopamine β-hydroxylase	Catecholamine production	Increased embryonic lethality, hypothalamic imbalance[b]
Hephaestin	Ferroxidase	X-linked iron deficiency anemia[b]
Lysyl oxidase	Cross-linking of collagen and elastin	Loose skin, hyperextensibility, vascular anomalies/aneurysms
Peptidylglycine α-amidating monooxygenase	Neuropeptide and peptide hormone processing	Unknown
Tyrosinase	Melanin synthesis	Depigmentation

[a] table contents compiled from Pena et al., 1999; Waggoner et al, 1999; Andrews et al, 2001

[b] deficiency phenotype identified in mice

oxygen species (ROS), such as the highly reactive hydroxyl radical (Pena et al., 1999). To address both the essentiality and toxicity of copper, organisms have developed homeostatic mechanisms that balance adequate copper acquisition with the prevention of toxic copper accumulation (Labbe and Thiele, 1999). Mechanisms designed to manage metal toxicity within the cell include strict control of metal uptake at the plasma membrane, intracellular metal sequestration, neutralization processes for ROS, and metal efflux systems (Askwith and Kaplan, 1998; Dameron and Harrison, 1998).

Experimental investigations using the yeast, *S. cerevisiae*, have rapidly advanced the study of metal metabolism in eucaryotic cells (Askwith and Kaplan, 1998). To date, studies in yeast have led to the identification of three independent intracellular copper transport pathways: the first delivers copper to the secretory pathway, the second transports copper to the Cu, Zn superoxide dismutase (Cu, Zn SOD), while the third shuttles copper to the mitochondrion (Figure 1-4) (Puig and Thiele, 2002).

Copper Uptake at the Plasma Membrane

Prior to or concomitant with high affinity uptake in *S. cerevisiae*, Cu(II) is thought to be reduced to Cu(I) at the cell surface by a number of Fe(III)/Cu(II) metalloreductases, including Fre1p, Fre2p, and possibly Fre7p (Labbe and Thiele, 1999). High affinity copper uptake in yeast has been shown to be mediated by two high affinity copper transporters, Ctr1p and Ctr3p (Pena et al., 1999). Ctr1p was originally isolated from a genetic screen used to identify yeast mutants with defects in iron (Fe) transport, demonstrating an inextricable link between copper and iron metabolism. Further investigation revealed that copper was required for the function of Fet3p, a Cu-dependent ferroxidase. Fet3p assembles with Ftr1p, a Fe permease, to form an Fe transport complex

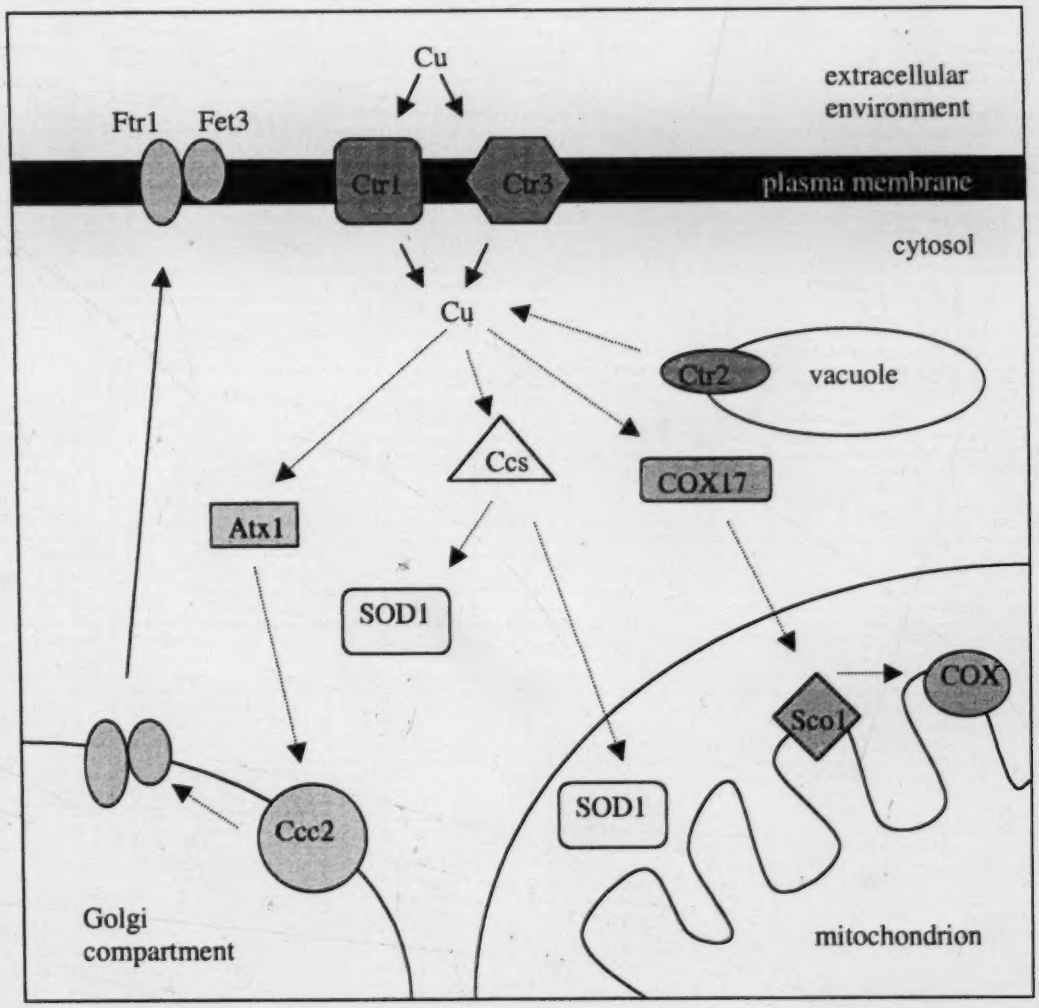


Figure 1-4. Copper uptake and distribution in *S. cerevisiae*. Copper is transported across the plasma membrane (from the extracellular environment) by the high-affinity copper transporters, Ctr1p and Ctr3p. Copper is distributed by an unknown mechanism to three different copper chaperones: Atx1p, CCSp, and Cox17p. Atx1p delivers copper to Ccc2p, a P-type ATPase located in the trans-Golgi network, which transports copper into the lumen of the Golgi for incorporation into the ferroxidase, Fet3p (Fet3p and Ftr1p, which are both involved in high affinity iron uptake, translocate to the plasma membrane). CCSp is responsible for transporting copper to Cu, Zn SOD, located in both the cytosol and the mitochondrial intermembrane space. Cox17p shuttles copper to Sco1p, a protein located in the mitochondrial inner membrane, for insertion into COX. Ctr2p may be involved in copper efflux from the vacuole. Modified from Puig and Thiele, 2002.

at the plasma membrane (Labbe and Thiele, 1999). Because Ctr2p can suppress the respiration-deficient phenotype of Δ*ctr1*Δ*ctr3* strains only in the presence of supplemental copper, it has been suggested that Ctr2p is involved in low-affinity Cu uptake (Puig and Thiele, 2002). Additional studies have also suggested that Ctr2p is localized in the vacuolar membrane in yeast cells and may be involved in the mobilization of stores of intracellular copper (Puig and Thiele, 2002).

Members of the Ctr1 family of high affinity copper transporters, which have subsequently been identified in plants and mammals, are plasma membrane proteins that contain several structurally conserved domains. These conserved regions include three putative transmembrane domains, an N-terminal portion that is rich in methionines, and a C-terminal region that contains well-conserved cysteines and histidines (Puig and Thiele, 2002). Although high affinity copper uptake has been shown to be an ATP-dependent process, none of the eucaryotic Ctr1 proteins have any features of ATPase domains (Pena et al., 1999). The exact mechanism that would explain how copper ions are transported across the plasma membrane by high affinity copper transporters remains to be elucidated.

The Cellular Requirement for Copper Chaperones

Once Cu crosses the plasma membrane, the potentially toxic heavy metal must be distributed to Cu-requiring enzymes and proteins in a non-reactive form (Labbe and Thiele, 1999). Using the Cu, Zn SOD copper delivery pathway as a model, Rae *et al.* have demonstrated that there is virtually no free copper in the yeast cell (Rae et al., 1999). To ensure an adequate supply of Cu to Cu-dependent proteins, the cell has evolved specialized proteins, known as Cu chaperones, to shuttle Cu to target enzymes

(Field et al., 2002). A specific copper chaperone has been identified in each of the known copper delivery pathways within the cell. Mutations in any one of the Cu chaperones causes a defect in its respective pathway, unlike mutations in the high-affinity copper transporters, which result in a pleiotropic phenotype due to a generalized cellular Cu deficiency (Labbe and Thiele, 1999). Although human homologues exist for each of Cu chaperones identified in yeast, no defects in Cu chaperone genes have been associated with human disease (Mercer, 2001).

Copper Delivery to the Secretory Pathway

Atx1p, the first copper chaperone identified in yeast, is a small, soluble protein that coordinates one atom of copper to the cysteines in a conserved MXCXXC motif (where X represents any amino acid) (Puig and Thiele, 2002). Copper is transferred from the conserved motif on Atx1p to an analogous site in the N-terminal region of Ccc2p (Pufahl et al., 1997). Structural studies indicate that Atx1p assumes a ferredoxin-like fold, which may protect bound copper from oxidants and competing thiols, such as glutathione (O'Halloran and Culotta, 2000).

Ccc2p and its mammalian homologues, ATP7A and ATP7B, are copper transporting P-type ATPases. Characteristic features of the P-type ATPases include a phosphorylation domain, a phosphatase domain, an ATP-binding site, and a transmembrane cation channel (Dameron and Harrison, 1998). Like Ccc2p, ATP7A and ATP7B are both localized to the trans-Golgi network (TGN). Both ATP7A and ATP7B, which show 57% amino acid identity, have eight transmembrane domains and six Cu-binding motifs. Although *ATP7A* and *ATP7B* are similar, the two genes have very different patterns of expression. While *ATP7A* is widely expressed, *ATP7B* is

predominantly expressed in liver and kidney (Cox, 1999). Mutations in *ATP7A* result in Menkes disease, an severe X-linked condition characterized by Cu deficiency, skeletal abnormalities, mental retardation, and neurologic deterioration (Chelly et al., 1993; Mercer et al., 1993; Vulpe et al., 1993). Clinical features of Wilson disease, which is caused by mutations in *ATP7B*, include reduced excretion of Cu into the bile, hepatic cirrhosis and neuronal degeneration (Bull et al., 1993; Tanzi et al., 1993). As with Atx1p and Ccc2p, ATOX1, the human homologue of Atx1, has been shown to directly interact with both ATP7A and ATP7B (Hamza et al., 1999; Larin et al., 1999).

Copper Delivery to Cu, Zn Superoxide Dismutase

Cu, Zn SOD, an antioxidant enzyme located in the cytosol and mitochondrial intermembrane space, uses Cu as a cofactor to catalyze the disproportionation of superoxide to hydrogen peroxide and oxygen (Puig and Thiele, 2002). CCSp (Copper Chaperone for SOD), the specific Cu chaperone for Cu, Zn SOD, binds one atom of Cu and is comprised of three distinct regions (Field et al., 2002). Domain I of CCS shares homology with Atx1p and contains an MXCXXC Cu-binding motif that may be involved in Cu acquistion. The second domain of CCS shares homology with Cu, Zn SOD and has been shown by the two-hybrid assay to be the region of interaction between the two proteins (Culotta et al., 1997; Casareno et al., 1998). Domain III of CCS contains a CXC motif and is the site of Cu binding and transfer to Cu, Zn SOD (Huffman and O'Halloran, 2001). Based on experimental evidence, it has been proposed that under Cu-limiting conditions, domain I of CCSp acquires Cu, which is then transferred to the Cu-binding site of domain III for incorporation into Cu, Zn SOD (Huffman and O'Halloran, 2001).

Mammalian homologues exist for both CCS and Cu, Zn SOD. As with mice that are deficient in Cu, Zn SOD, mice in which *Ccs* has been disrupted by targeted deletion are viable, but show severely reduced Cu, Zn SOD activity and a sensitivity to the superoxide-generating drug, paraquat (Wong et al., 2000). While Cu, Zn SOD deficiency has not been identified as a pathological condition in humans, gain-of-function mutations in the gene encoding Cu, Zn SOD have been associated with subset of cases of familial amyotrophic lateral sclerosis (FALS) (Waggoner et al., 1999). FALS is neurogenerative disorder characterized by progressive muscle weakness, atrophy, and death, often within a few years of onset.

Copper Delivery to the Mitochondrion

(i) The mitochondrial copper chaperone

Historically, COX has been the only known mitochondrial cuproenzyme. Cox17p, a small, hydrophilic, cysteine-rich molecule, was the first protein identified to have a role in the provision of copper to COX (Glerum et al., 1996a). The suggestion that Cox17p is involved in mitochondrial copper transport came from the observation that the growth of a Δ*cox17* strain on nonfermentable carbon sources could be restored when the medium was supplemented with copper. In the Δ*cox17* strain, neither the copper,zinc-superoxide dismutase (Cu,Zn-SOD) nor the high-affinity copper transporter Ctr1p were affected, indicating that Cox17p is part of a mitochondrial-specific copper pathway (Glerum et al., 1996a). Cox17p has a dual localization, with 60% of the protein found in the mitochondrial intermembrane space and 40% of the protein localized to the cytosol (Beers et al., 1997). Lacking both a classical N-terminal mitochondrial targeting

sequence and intermembrane sorting presequence, it has been suggested that Cox17p is internalized in the mitochondrion by a unique mechanism (Beers et al., 1997).

Based on the cysteine content of the protein, Cox17p could be expected to bind two to three copper atoms per molecule of protein (Beers et al., 1997). Although studies have demonstrated that Cox17p is in fact a cuproprotein, it is currently unclear how many copper ions it binds. Studies of Cox17p purified from yeast have indicated that the protein binds two ions of copper (Beers et al., 1997), while yeast Cox17p isolated from *E. coli* binds two (Srinivasan et al., 1998) or three (Heaton et al., 2001) copper ions. Ultraviolet absorption spectroscopy studies have indicated that copper is bound to Cox17p via thiolate ligands (Srinivasan et al., 1998). The luminescence of copper-bound Cox17p suggests a Cu(I) coordination in a solvent-shielded environment, although the copper is much more solvent accessible than the copper bound to the yeast metallothionein, Cup1p (Srinivasan et al., 1998). Copper is not tightly bound to Cox17p (Beers et al., 1997) and can be rapidly displaced in the presence of the copper chelator, bathocuproinedisulfonic acid (BCS) (Srinivasan et al., 1998).

Cox17p contains seven cysteine residues, six of which are conserved among all eucaryotic COX17 proteins (Figure 1-5). Mutational analysis of the cysteine residues in Cox17p suggested that only Cys23, Cys24, and Cys26, the residues found in the putative KPCCXC copper-binding motif, are essential for function (Heaton et al., 2000). When one essential cysteine residue is mutated, Cox17p still binds copper to the same extent as the wild-type protein. In the presence of two mutated cysteine residues, however, copper binding is abolished (Heaton et al., 2000). Because the distribution of Cox17p in the mutants in which copper binding has been abrogated is unaltered relative to the wild-type

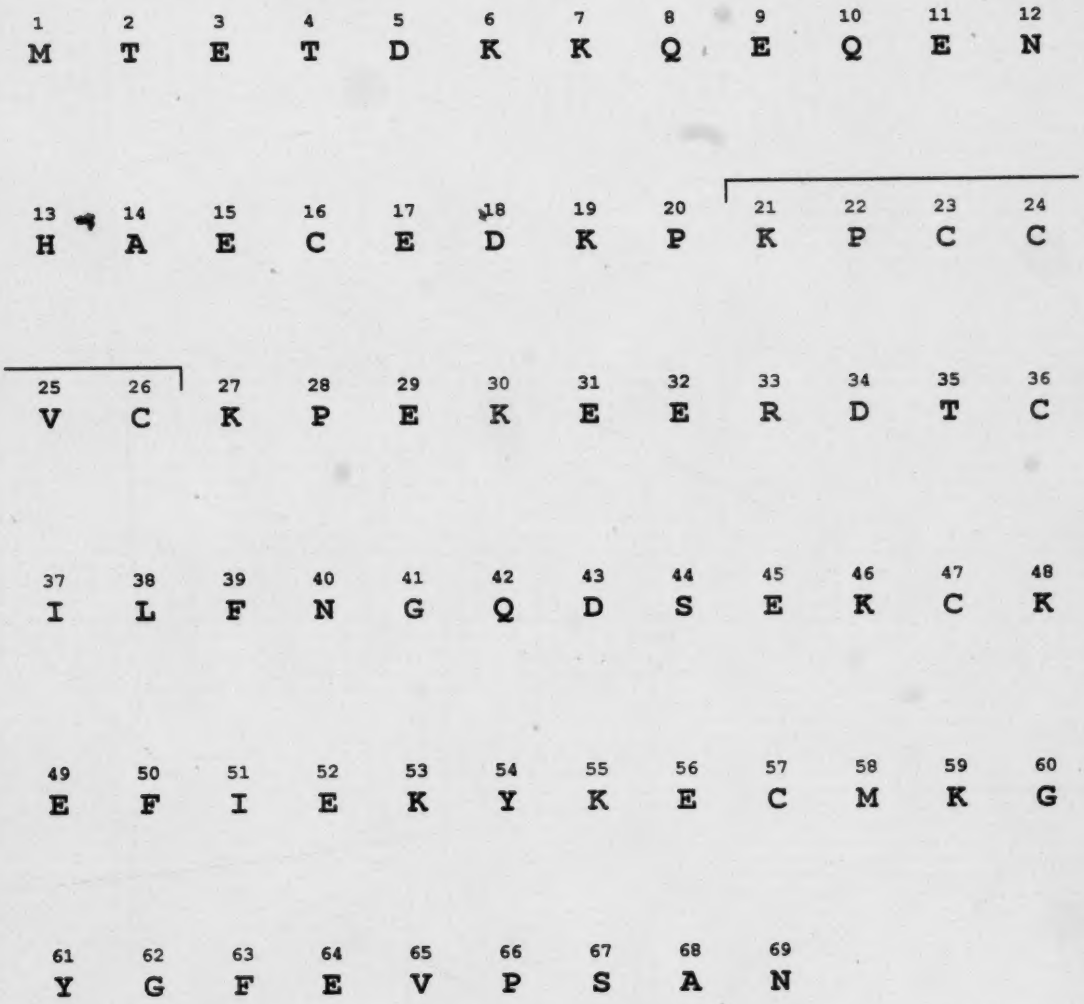


Figure 1-5. The amino acid sequence of yeast Cox17p. Amino acids that are conserved between yeast and human COX17 proteins are shown in blue. The copper-binding motif is indicated by a black bracket above the sequence.

protein, it has been suggested that mitochondrial localization is independent of copper binding (Heaton et al., 2000).

Using gel filtration techniques, Heaton *et al.* (Heaton et al., 2001) isolated at least three species containing Cox17p, suggesting that the protein is found in oligomeric complexes. Based on the findings of the gel filtration study, it was proposed that the Cox17p in the intermembrane space is predominantly tetrameric, whereas the Cox17p in the cytosol would be dimeric. However, the Cox17p purified in the gel filtration experiments was expressed in *E. coli*, a heterologous system that does not contain mitochondria. The oligomeric status of yeast Cox17p remains to be determined in its native environment.

The human *COX17* cDNA has been isolated by functional complementation of a yeast *cox17* mutant and encodes a 63 amino acid protein (Amaravadi et al., 1997). Dopuin, which means "many proline factor" in Chinese, is the porcine version of COX17 and has been chemically characterized from pig small intestine (Chen et al., 1997). It was noted at the time of isolation that the protein has several interesting features, including a large concentration of proline residues in the N-terminal portion, three histidine residues spaced throughout the C-terminal half, and three disulfide bridges in the central segment of the protein. The purpose of the proline residues in the mammalian COX17 proteins is uncertain as these residues are not conserved in yeast Cox17p.

Cox17 cDNAs have also been isolated from mouse and rat (Nishihara et al., 1998; Kako et al., 2000). In both animals, *Cox17* was found to be ubiquitously expressed in all tissues tested. Characterization of the murine *Cox17* promoter shows that its proximal region is GC-rich and does not contain TATA or CAAT box motifs, characteristic of a

housekeeping gene (Takahashi et al., 2001). Electrophoretic mobility shift assays using serial deletion constructs of the promoter region indicate that the basal transcription of *Cox17* may be under the control of the Sp-1 and NRF-1 transcription factors, similar to other COX-related genes (Takahashi et al., 2002a). More recently, a transgenic mouse with a disruption in *Cox17* has been generated (Takahashi et al., 2002b). While heterozygous mice were healthy, the homozygous gene disruption was embryonic lethal, with death occurring between E8.5 and E10. Embryos obtained at E6.5 had severely reduced COX activity. The embryonic lethality of the *Cox17* knock-out mouse suggests that mammalian COX17 has an essential role in COX function and embryonic development (Takahashi et al., 2002b).

(ii) A comparison of the cellular copper chaperones

Both Atx1p and CCSp contain a conserved MXCXXC Cu-binding motif, while Cox17p contains a KPCCXC motif. Atx1p and CCSp are both able to bind only one atom of Cu, while Cox17p can coordinate at least two atoms of copper. Finally, Atx1p and CCSp share a homologous domain with their respective target proteins, while Cox17p does not appear to bear any homology to Sco1p. While the reason for the differences between Cox17p and the other two Cu chaperones is unknown, a greater understanding of mitochondrial copper acquisition may shed some light on the unique features of Cox17p, the putative mitochondrial Cu chaperone.

(iii) Intramitochondrial copper delivery

SCO1, encoding a mitochondrial inner membrane protein (Schulze and Rodel, 1988; Schulze and Rodel, 1989; Buchwald et al., 1991) originally suggested to be involved in the assembly of COXI and COXII into the COX holoenzyme (Krummeck

and Rodel, 1990), was isolated as a multicopy suppressor of a *cox17* mutant (Glerum et al., 1996b). When overexpressed, Sco1p is capable of rescuing a defect in Cox17p, leading to the proposal that Sco1p acts downstream of Cox17p in the copper recruitment pathway (Glerum et al., 1996b). Unlike *cox17* mutants, defects in Sco1p cannot be rescued by supplemental copper, nor by the overexpression of Cox17p or the high affinity copper transporter, Ctr1p (Glerum et al., 1996b). A second protein, Sco2p, was also shown to act as a high copy suppressor of a point mutation in *cox17*, although incapable of suppressing a *cox17* null mutation. While Sco1p and Sco2p are closely related proteins, only Sco1p is required for respiration on nonfermentable carbon sources. The lack of phenotype in the Δ*sco2* yeast strain suggests that there may be some redundancy between the two proteins, with partial overlap in function (Glerum et al., 1996b).

Sco1p contains an N-terminal mitochondrial targeting sequence, which is proteolytically processed after import into the mitochondrial matrix (Beers et al., 2002). The single hydrophobic transmembrane domain of Sco1p is believed to function as a stop-transfer signal (Beers et al., 2002), such that the C-terminal portion of the protein, including the putative copper-binding motif, remains exposed in the mitochondrial intermembrane space (Rentzsch et al., 1999). CXXXC, the putative copper-binding motif found in Sco1p, is reminiscent of the copper-binding motif found in COX subunit II (Glerum et al., 1996b). Mutation of either cysteine residue in the motif results in a respiration-deficient phenotype, indicating that both residues are required for Sco1p function (Rentzsch et al., 1999). Purified Sco1p has been shown to bind one copper atom per molecule of protein, with binding occurring with much higher affinity than in Cox17p (Nittis et al., 2001; Beers et al., 2002). X-ray absorption spectroscopy data suggest that

Cu(I) has a trigonal coordination when bound to Sco1p, that the ligation complex consists of two Cu-S and one Cu-N, and that the Cys148 and Cys152 residues in the copper-binding motif are involved in Cu(I) ligation (Nittis et al., 2001).

Both *in vitro* and *in vivo* experiments have demonstrated a physical interaction between Sco1p and COX subunit II (Lode et al., 2000). These studies indicated that the interaction is not dependent on the presence of copper, nor is the interaction mediated by Sco2p. Using affinity chromatography, it was shown that Sco1p can bind itself (Lode et al., 2000), which agrees with the sedimentation analysis studies that found that Sco1p is a homotrimer (Beers et al., 2002). Immunoprecipitation experiments showed that Sco2p, which also forms homomers, can be found in a complex with COX subunit II (Lode et al., 2002). In vitro binding assays have also demonstrated that Sco1p and Sco2p interact, forming heteromeric complexes. Although it has been suggested that Sco2p plays a role in COX assembly or stability (Lode et al., 2002), the protein is dispensable for respiratory growth and its function within the mitochondrion remains unclear.

The two *SCO* genes found in humans, *SCO1* and *SCO2*, do not functionally complement a yeast *sco1* mutant (Paret et al., 1999). Analysis of *SCO* genes in yeast and humans suggest that the two *SCO* genes found in each organism arose by independent gene duplications in each of the two lineages (Papadopoulou et al., 1999). Human *SCO1* is an ubiquitously expressed gene on chromosome 17p12-p13, which encodes a mitochondrial protein sharing 34% identity with yeast Sco1p (Petruzzella et al., 1998). Studies of a large family with multiple cases of isolated COX deficiency, neonatal-onset hepatic failure, and encephalopathy revealed mutations in human *SCO1* (Valnot et al.,

2000a). In the affected individuals, analysis of COX activity in a variety of tissues demonstrated a severe COX deficiency in liver, skeletal muscle, and lymphocytes.

Mutations in human *SCO2* have been found in association with isolated COX deficiency and fatal infantile cardioencephalomyopathy (Papadopoulou et al., 1999; Jaksch et al., 2000). Although *SCO2*, which is located on chromosome 22q13, is ubiquitously expressed, highest levels of expression are seen in heart, skeletal muscle, brain, liver, and kidney (Papadopoulou et al., 1999). Patients with mutations in *SCO2* show a severe reduction in COX activity in heart and skeletal muscle, and a severe reduction in COX subunits I and II (Papadopoulou et al., 1999). To understand the effect of the mutations on the function of the protein, the two first mutations found in human *SCO2* were introduced into yeast Sco1p. While the E155K mutation (E140K in human SCO2) did not affect Sco1p function, the S240F mutation (S225F in human SCO2) resulted in a yeast strain that was respiration deficient with greatly reduced COX activity, a specific loss of subunit II, and misassembled COX (Dickinson et al., 2000). In the S240F mutant, the α band, which is due to the absorbance of the *a* and a_3 cytochromes, was shifted from its normal position at 603 nm towards the blue. The S240F strain described represented the first yeast COX mutant described with a partial assembly phenotype. Because COX subunit II is specifically missing, Dickinson *et al.* (Dickinson et al., 2000) suggest that Sco1p is involved in copper insertion at the Cu_A site of subunit II. Furthermore, since COX is partially assembled, the authors propose that Sco1p functions late in the COX assembly pathway (Dickinson et al., 2000).

In a study of the human SCO2, atomic absorption spectroscopy and ion-coupled plasma atomic emission spectroscopy were used to show that the protein binds copper

(Jaksch et al., 2001). The incubation of patient myoblasts with increasing amounts of copper-histidine resulted in increasing levels of COX activity, as well as increasing amounts of COX subunit II. A copper uptake study demonstrated that patient fibroblasts had increased concentrations of cellular copper, suggesting a compensatory mechanism in cells with a defect in mitochondrial copper metabolism. Because of a previous suggestion that Sco1p and Sco2p share similarities with thiol:disulfide oxidoreductases with a thioredoxin fold (Chinenov, 2000), SCO2 was tested for its ability to reduce disulfide bridges. Using a classical thioredoxin assay, Jaksch *et al.* could not demonstrate that the protein was capable of reducing disulfide bonds, suggesting that human SCO2 does not function as a disulfide reductase (Jaksch et al., 2001).

A potential homologue of yeast Sco1p, YpmQ, has been identified in the bacterium, *B. subtilis* (Mattatall et al., 2000). *B. subtilis* has two terminal oxidases, including a cytochrome *c* oxidase, which contains both Cu_A and Cu_B, and a menaquinol oxidase, which contains Cu_B only. YpmQ has a single transmembrane domain, shares 27% identity with Sco1p in its soluble domain, and contains the CXXXC motif and conserved histidine residue found in Sco1p. The Δ*ypmQ* strain has a similar phenotype to the *sco1* null mutant strain, exhibiting a loss of spectral properties, COX activity, and major COX subunits. Because the function of menaquinol oxidase is unaffected when *YpmQ* is disrupted, Mattatall *et al.* suggest that YpmQ is involved in the assembly of Cu_A, but not Cu_B, in COX biogenesis in *B. subtilis* (Mattatall et al., 2000).

The similarity between the phenotypes of yeast strains with mutations in *COX10* and *COX11*, which is homologous to *ORF3* of the COX operon in *P. denitrificans*, originally suggested that the genes encoded proteins with related functions (Tzagoloff et

al., 1990). Subsequent studies, however, have implicated the *COX11* gene product in mitochondrial copper metabolism, rather than heme A biosynthesis. Like Sco1p, Cox11p is a mitochondrial protein with an N-terminal mitochondrial targeting sequence and a single transmembrane domain. Purified yeast Cox11p has been shown to bind one atom of copper per molecule of protein, with Cu(I) ligated by three sulfurs. Mutations introduced into the three cysteines in the globular domain of Cox11p render the yeast respiration deficient and abolish copper binding in the protein (Carr et al., 2002).

Like yeast *COX11*, the *COX11* gene found in *R. sphaeroides* encodes a protein with a single transmembrane helix and a large globular domain (Hiser et al., 2000). When *COX11* is disrupted, spectral analysis of the bacterial strain demonstrates a 3-4 nm shift to the blue in both the α and the Soret bands, indicating altered environments around both heme centres. Heme A content in the mutant strain is normal, as are COX subunit I-III levels, suggesting that Cox11 is not required for the insertion of heme A or the assembly of the catalytic COX subunits. EPR analysis of the Δ*cox11* strain confirms that the heme A environment is disturbed, and also indicates that while Cu_A is unaffected, Cu_B is absent. When magnesium is substituted for the EPR-visible manganese in the mutant strain, EPR also demonstrates that much less manganese is present in the mutant relative to the wild-type strain. Hiser *et al.* speculate that Cox11 normally functions in the insertion of Cu_B into subunit I of COX, and that the presence of Cu_B is likely to facilitate the stable formation of other structures in subunit I (Hiser et al., 2000). Because COX is assembled to a high degree in the cytoplasmic membrane of the Δ*cox11* strain, Cu_B insertion may be a late step in COX assembly, occurring after heme A insertion and the association of subunit I with subunits II and III. Phenotypic differences between *cox11*

null mutants in *S. cerevisiae* and *R. sphaeroides*, which has a milder defect than the yeast, may be due to the presence of a more complicated enzyme in yeast (Hiser et al., 2000).

The human *COX11* gene is ubiquitously expressed and localizes to chromosome 17q22 (Petruzzella et al., 1998). The N-terminus of COX11 has the typical features of a mitochondrial leader peptide, and the COX11 protein is imported into the mitochondrion. Human COX11 has 41% amino acid identity with its yeast counterpart. In addition to the expressed version of *COX11*, a putative pseudogene has also been identified on chromosome 6p22-p23. To date, no mutations in *COX11* have been implicated in human COX deficiency.

MITOCHONDRIAL DISEASE

The term, mitochondrial disease, is generally used in reference to the phenotypically diverse group of disorders that result from defects in mitochondrial energy production (Zeviani, 2001). Because OXPHOS is the only metabolic pathway that falls under the dual genetic control of the mitochondrial and nuclear genomes (DiMauro, 2001b), an OXPHOS defect may present with any clinical manifestation, affect any organ or tissue, occur at any age, and display any mode of inheritance (Munnich and Rustin, 2001). The clinical heterogeneity seen in mitochondrial disorders can be explained in part by the fact that virtually all cells depend on mitochondrial OXPHOS for the generation of ATP (Simon and Johns, 1999). Not surprisingly, however, some tissues appear to be more sensitive to mitochondrial dysfunction, including those with higher aerobic demand, such as skeletal muscle, brain, and heart (Di

Donato, 2000). In general, mitochondrial diseases manifest as pure myopathies or as multisystem disorders (van den Heuvel and Smeitink, 2001).

The first report of an OXPHOS disease appeared in 1962 (Luft, 1962), describing a rare condition now known as Luft disease (Shoffner, 2001). Since the 1960's, the catalogue of mitochondrial disorders has greatly expanded, with the incidence of mitochondrial disease estimated at 1 in 10 000 live births (Bourgeron et al., 1995). Until quite recently, the search for etiological mutations in OXPHOS diseases has focused primarily on the mitochondrial genome. However, since the majority of OXPHOS disorders are transmitted as autosomal recessive traits, it is evident that a significant proportion of pediatric and adult OXPHOS disorders are the result of mutations in the nuclear genome (Shoffner, 2001).

Currently, there is no treatment available for mitochondrial diseases and management of these disorders is limited to the palliative administration of vitamins and cofactors (DiMauro, 2001b). Although the purpose of these agents is to modify respiratory chain function or reduce cytotoxic metabolites and free radicals, the therapeutic benefits of cofactor treatment are unknown because the studies are largely anecdotal or based on small clinical trials (Taylor et al., 1997).

Mitochondrial Disease due to Mutations in Mitochondrial DNA

The mitochondrion was first recognized as containing DNA in 1963 (Nass and Nass, 1963), although the sequence of the mitochondrial genome did not become available until 1981 (Anderson et al., 1981). The first identification of mutations in mtDNA occurred seven years later with the report of deletions in muscle mtDNA causing mitochondrial myopathies (Holt et al., 1988) and a point mutation in the subunit 4 gene

of complex I leading to Leber's hereditary optic neuropathy (LHON), a maternally-inherited disorder that results in acute bilateral blindness (Wallace et al., 1988).

The mitochondrial genome is a small, circular, double-stranded molecule, consisting of 16 658 base pairs (bp) of DNA (Smeitink et al., 2001) (Figure 1-6). Mitochondrial DNA is present at 10^3 to 10^4 copies per cell, with an estimated 2 to 10 copies of mtDNA per mitochondrion (Smeitink et al., 2001). Thirty-seven genes are found in mtDNA, including two ribosomal RNA genes (12S and 16S rRNAs), 22 transfer RNAs (tRNAs), and 13 protein-coding genes, which encode subunits of four of the OXPHOS enzymes. Mammalian mtDNA shows extreme economy of organization, with no introns, few or no noncoding bases between adjacent genes, one regulatory region (known as the D-loop), small tRNA and rRNA molecules, and some overlapping protein-coding genes (Taanman, 1999). The mammalian mitochondrial genome also contains a simplified genetic code, which is read using a minimal set of 22 tRNAs *versus* the 32 tRNAs required for Crick's wobble hypothesis (Anderson et al., 1981). The mutation rate of mtDNA is ten times greater than that of the nuclear genome, in part because the mitochondrial genome contains no protective histones and has no effective DNA repair system, making it very susceptible to free radical damage. Because mtDNA does not contain any introns, mutations arising in the mitochondrial genome are likely to affect coding sequence (Taylor et al., 1997).

Several distinctive characteristics of mitochondrial genetics, including maternal inheritance, heteroplasmy and threshold effect, and mitotic segregation, confer unique features to mitochondrial disease due to mutations in mtDNA. Maternal inheritance of mtDNA mutations will almost always result in a pedigree in which the trait is passed

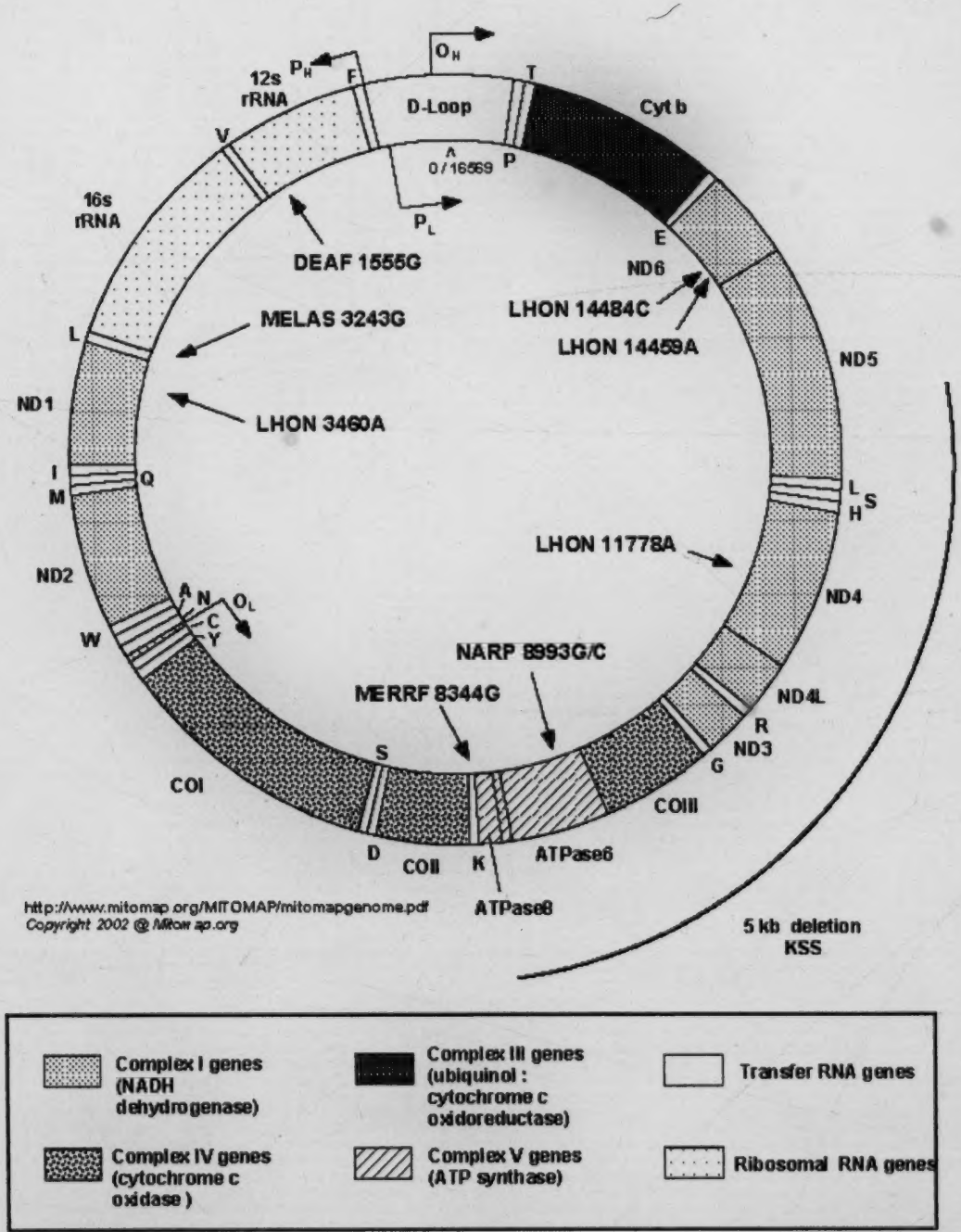


Figure 1-6. Schematic of the human mitochondrial genome. Human mitochondrial DNA is a circular, double-stranded molecule consisting of approximately 16.6 kb of DNA. Common mutations associated with mitochondrial disease are indicated by arrows. Source of figure: http://www.mitomap.org.

from mother to offspring. When two populations of mtDNA, mutant and wild-type, are present together, a tissue, and therefore, an individual, is said to be heteroplasmic. If the proportion of mutant to wild-type mtDNAs exceeds a certain threshold level within a tissue, clinical manifestation of disease will occur, which is known as the threshold effect. At cell division, the distribution of mutant and wild-type mtDNAs in the daughter cells may be altered relative to the parent cell. This phenomenon, known as mitotic segregation, explains how the mutant load of a cell can be altered, changing the phenotype of an individual accordingly (DiMauro, 2001b).

The mtDNA mutation underlying a mitochondrial disease is often difficult to infer from the clinical presentation of the patient, since the same mutation can cause disparate phenotypes in different individuals and the same phenotype can be caused by mutations in different genes (Leonard and Schapira, 2000). In general, the two types of mutations that arise in mtDNA include mtDNA rearrangements, which tend to occur sporadically, and point mutations, which usually are maternally-inherited (DiMauro and Schon, 2001). More than 100 different mtDNA rearrangements have now been detected in patients with mitochondrial disease (Chinnery and Turnbull, 1999). Common mitochondrial disorders attributed to deletions of mtDNA include chronic progressive external ophthalmoplegia (CPEO), Kearns-Sayre syndrome (KSS), and Pearson syndrome (DiMauro, 2001a). CPEO is the most common manifestation of an OXPHOS mtDNA defect and is characterized by onset in the second or third decade of life and a relatively benign clinical course (Leonard and Schapira, 2000). When the onset of CPEO occurs during adolescence and in conjunction with symptoms such as sensorineural deafness, cerebellar ataxia, retinitis pigmentosa, diabetes mellitus, and cardiac conduction defects, the disease

is said to be KSS, which is a devastating progressive neurological disorder (Chinnery and Turnbull, 1999). Pearson syndrome represents the most severe mtDNA rearrangement syndrome (Wallace, 2001a) and is characterized by sideroblastic anemia or pancytopenia with exocrine pancreatic failure. Additional clinical features include progressive liver disease, diabetes mellitus, and renal tubular dysfunction. Onset occurs during infancy and the prognosis is usually very poor (Leonard and Schapira, 2000). Those individuals that survive the pancytopenia progress to develop KSS (Wallace, 2001a).

Point mutations arising in mtDNA may affect protein synthesis (occurring in a tRNA or rRNA gene), or may result in a defective OXPHOS subunit (occurring in a protein-coding gene). Although tRNA genes represent approximately 10% of total mtDNA, mutations in tRNA genes account for approximately 75% of mtDNA-related disorders (Smeitink et al., 2001). Common syndromes due to point mutations in tRNA genes include the two encephalomyopathies, myoclonic epilepsy with ragged red fibres (MERRF) and mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS). MERRF usually presents in late adolescence or early adulthood, has a variable prognosis, and may include myopathy, ataxia, and deafness (Leonard and Schapira, 2000). MELAS, which is probably the most common of the mitochondrial encephalomyopathies, may be associated with deafness, diabetes, seizures, dementia, and ataxia. The onset of MELAS normally occurs during childhood or adolescence (Leonard and Schapira, 2000). Pathogenic mutations in mitochondrial rRNA genes are rare, but have been described in individuals with cardiomyopathy or deafness (DiMauro, 2001a).

Point mutations in protein-coding OXPHOS genes can lead to a multitude of clinical phenotypes, including many different myopathies and encephalopathies, or a

combination of the two (encephalomyopathies). Two common disorders that result from a mutation in a mtDNA protein-coding gene include neuropathy, ataxia, and retinitis pigmentosa (NARP), and maternally inherited Leigh syndrome (MILS). Leigh syndrome (including the maternally-inherited form) is an early-onset, fatal neurodegenerative disorder characterized by bilaterally symmetrical necrotic lesions in the brain (DiMauro and De Vivo, 1996). Clinical features include hypotonia, psychomotor regression, seizures, myoclonus, ataxia, brainstem dysfunction, optic atrophy, and peripheral neuropathy (Simon and Johns, 1999). Death usually occurs within two years of onset (Dahl, 1998). NARP and MILS are due to the same heteroplasmic mutation in *ATP6*, which encodes a subunit of complex V. When the proportion of mutant mtDNA present is 80 to 90% of total mtDNA, the disease manifests as NARP. If the mutational burden exceeds 90 to 95%, the more severe MILS will develop (Manfredi et al., 2002).

With no effective treatment currently available for mitochondrial disorders, it has been suggested that genetic strategies offer the most hope towards developing useful therapies (Taylor et al., 1997). Gene therapy for mitochondrial diseases due to defects in mtDNA present additional challenges beyond those that are faced in nuclear gene therapy. Each cell contains multiple copies of mtDNA, many mutations are heteroplasmic, and the therapeutic agent must be able to enter the mitochondrion (Chinnery and Turnbull, 1999). One strategy that has been proposed to correct defects in mtDNA protein-coding genes is to express the mitochondrial gene within the nucleus, such that its gene product is translated in the cytosol and targeted to the mitochondrion (Taylor et al., 1997). The rescue of a mutant mitochondrial gene by a wild-type copy expressed in the nucleus has been carried out successfully in yeast and more recently in a

mammalian cell line using *ATP6* (Manfredi et al., 2002). A second strategy that has been proposed for the correction of mtDNA mutations is the use of peptide nucleic acids to specifically inhibit the replication of mutant mtDNA. This approach would allow wild-type mtDNA to repopulate the cell, reducing the proportion of mtDNA below the clinical threshold (Taylor et al., 1997). In addition to the gene therapy strategies, a treatment that has been proposed for myopathies involves the injection of a toxin into affected muscles to induce necrosis. Satellite cells, which are myogenic precursor cells and tend to contain little or no mutant mtDNA, will proliferate in response to acute muscle damage. It is thought that the repopulation of muscle fibres with a higher percentage of normal mtDNA will decrease the mutational burden and reverse the biochemical and genetic defects associated with mitochondrial myopathies (Simon and Johns, 1999).

Mitochondrial Disease Due to Mutations in Nuclear DNA

Mitochondria are thought to contain more than one thousand different proteins (Dahl and Thorburn, 2001). With only thirteen OXPHOS polypeptides encoded by the mtDNA, the remaining proteins are encoded by nDNA, illustrating the importance of the nuclear genome in mitochondrial disease. Although many mitochondrial disorders have long been suspected to be due to defects in nuclear genes, given their autosomal inheritance patterns, the identification of underlying mutations has been a much slower process than the detection of mtDNA mutations. This is partly because the mitochondrial genome is extremely small relative to its nuclear counterpart, and because it was not recognized early on that the majority of mitochondrial disorders have clear Mendelian inheritance or that the mitochondrial respiratory chain is so complex (Di Donato, 2000). It has been suggested that the identification of nuclear genes involved in OXPHOS

defects in the future will be most fruitful by taking a functional complementation approach, such as microcell-mediated chromosome transfer or cDNA expression libraries (Shoubridge, 2001b).

One interesting feature of mitochondrial disease caused by mutations in nDNA is the paucity of mutations found in nuclear genes coding for the structural subunits of OXPHOS complexes. The majority of nuclear genetic lesions identified to date have been found in genes that regulate OXPHOS processes, such as OXPHOS subunit assembly (Shoffner, 2001). The large but unknown number of proteins involved in the assembly and maintenance of OXPHOS complexes represent potential defects that could be underlying causes of mitochondrial disease (Shoubridge, 2001b).

In addition to the many nuclear genes that regulate different aspects of OXPHOS complex biogenesis, the nuclear genome also provides all of the factors responsible for mtDNA replication and expression (Hirano et al., 2001). Defects in intergenomic communication cause both qualitative and quantitative abnormalities of mtDNA (Suomalainen and Kaukonen, 2001). Two different mitochondrial disorders, autosomal dominant progressive external ophthalmoplegia (adPEO) and mitochondrial neurogastrointestinal encephalomyopathy (MNGIE), have been shown to be the result of primary mutations in nuclear genes that cause secondary deletions or depletion of mtDNA (Suomalainen and Kaukonen, 2001). AdPEO is typically characterized by progressive ophthalmoplegia with multiple mtDNA mutations in skeletal muscle, but symptoms may also include proximal limb weakness, severe depression, peripheral neuropathy, sensorineural hearing loss, cataracts, and endocrine dysfunction (Hirano and DiMauro, 2001). Three different nuclear genes have been implicated in adPEO,

indicating that it is a genetically heterogeneous disorder. Mutations causing adPEO were first found in *ANT1*, which encodes the heart and skeletal muscle-specific isoform of the adenine nucleotide translocator (Kaukonen et al., 2000). More recently, lesions in *POLG*, which encodes the mtDNA polymerase γ, have been shown to cause adPEO (Van Goethem et al., 2001), and represent the first genetic defects found in the mtDNA replication machinery (Hirano et al., 2001). Mutations in *C10orf2*, whose gene product is a protein called Twinkle, are also known to cause adPEO (Spelbrink et al., 2001). Twinkle has homology to a T7 bacteriophage helicase/primase, which has led to the proposal that Twinkle is the mtDNA helicase (Spelbrink et al., 2001). It is not clear how defects in the three nuclear-encoded mitochondrial proteins implicated in adPEO lead to the disease phenotype, but it appears that they all play a role in the maintenance of mtDNA integrity. The clinical phenotype of MNGIE is characterized by the unusual combination of progressive ophthalmoplegia, severe gastrointestinal dysmotility, cachexia, peripheral neuropathy, leukoencephalopathy, and mitochondrial dysfunction (Hirano et al., 2001). MNGIE is an autosomal recessive disorder with variable age of onset, and has been found to result from mutations in the thymidine phosphorylase gene, *TP* (Nishino et al., 1999). Thymidine phosphorylase is involved in the phosphorylytic catabolism of the nucleoside thymidine to thymine. It has been hypothesized that an imbalance in mitochondrial deoxynucleotide pools leads to impaired mtDNA synthesis, producing the mtDNA depletion and deletions seen in MNGIE patients (Hirano et al., 2001). Mutations leading to autosomal recessive mtDNA depletion syndromes have also been found in two deoxyribonucleoside kinases, which are involved in the nucleotide salvage pathway in mitochondria (Elpeleg et al., 2002). Defects in the gene encoding the

mitochondrial deoxyguanosine kinase have been implicated in a hepatocerebral mtDNA depletion syndrome, which is characterized by early progressive liver failure, neurological abnormalities, hypoglycemia and decreased activity of mtDNA-encoded respiratory chain complexes (Mandel et al., 2001). A severe myopathy with muscle-specific depletion of mtDNA and reduced activity of mtDNA-encoded respiratory chain complexes has been shown to result from mutations in the mitochondrial thymidine kinase gene (Saada et al., 2001). As in MNGIE, it appears that the underlying pathology of these two intergenomic communication disorders is related to a disturbance of the mitochondrial deoxynucleotide pools and that these nuclear-encoded factors play a critical role in maintaining normal levels of mtDNA (Elpeleg et al., 2002).

Mitochondrial Disease due to Mutations in Nuclear Genes, Leading to Secondary OXPHOS Defects

In recent years, a number of mitochondrial disorders stemming from defects in nuclear DNA have been identified in which the disease phenotypes are not associated with overt OXPHOS defects. The proteins that are defective in these disorders play a role in a mitochondrial metabolic pathway that is indirectly related to energy production. Although a specific OXPHOS defect is not considered to be the primary cause of disease, abnormalities in the respiratory chain may contribute to the pathogenesis of these disorders (Zeviani, 2001). Mitochondrial diseases with secondary OXPHOS defects include Friedreich ataxia, Mohr-Tranebjerg syndrome, hereditary spastic paraplegia, autosomal dominant optic atrophy, and X-linked sideroblastic anemia with ataxia. Friedreich ataxia (FA) is the most common hereditary ataxia and is inherited in an autosomal recessive fashion. Clinical features of FA include degeneration in the central

and peripheral nervous system, cardiomyopathy, skeletal abnormalities, and increased risk of diabetes (Pandolfo, 2002). The majority of patients have an unstable GAA trinucleotide repeat expansion in the first intron of the FA gene, which encodes frataxin (Campuzano et al., 1996). Loss of frataxin, which localizes to the mitochondrion (Priller et al., 1997), causes mitochondrial iron accumulation, deficiency of Fe-S cluster proteins (including OXPHOS complexes I-III), mitochondrial dysfunction, and increased oxidative stress (Pandolfo, 2002). The physiological role of frataxin remains controversial, but studies in mammals and yeast suggest that frataxin may function in mitochondrial iron homeostasis, Fe-S cluster biogenesis, or directly in the response to oxidative stress (Puccio and Koenig, 2002).

Mohr-Tranebjaerg syndrome, also known as deafness dystonia syndrome, is an X-linked disease that arises from mutations in the DDP1 gene (Jin et al., 1996). The syndrome is characterized by progressive sensorineural deafness, progressive dystonia, spasticity, dysphagia, mental deterioration, paranoia, and cortical blindness. DDP1 is a mitochondrial intermembrane space protein and is homologous to yeast Tim8p, which interacts with the protein import system of the mitochondrial inner membrane (Koehler et al., 1999). Mohr-Tranebjaerg syndrome is the first example of a human disease associated with a defect in mitochondrial protein import (Zeviani, 2001).

One form of hereditary spastic paraplegia (HSP), which is characterized by progressive lower extremity weakness and spasticity, has been found to be due to defects in *SPG7* (Casari et al., 1998). The *SPG7* gene product, known as paraplegin, is a mitochondrial protein that is highly homologous to a subclass of yeast mitochondrial metalloproteases involved in the turnover of mitochondrial inner membrane proteins

(Shoubridge, 2001b). Muscle biopsy specimens from patients diagnosed with HSP show typical signs of OXPHOS impairment (Casari et al., 1998).

Dominant optic atrophy is the most prevalent inherited optic neuropathy, typically presenting in childhood with bilateral visual loss (Votruba et al., 1998). Causative mutations have been found in the *OPA1* gene, which encodes a dynamin-related protein localized to mitochondria (Delettre et al., 2000). In yeast, dynamin-related proteins have been shown to play an important role in the maintenance and inheritance of mitochondria (Alexander et al., 2000). It has been suggested that defects in human *OPA1* affect mitochondrial integrity, leading to the impairment of mtDNA maintenance and mitochondrial energy production (Delettre et al., 2000).

Defects in *ABC7*, which encodes an ATP-binding cassette transporter, have been implicated in X-linked sideroblastic anemia and ataxia (XLSA/A) (Allikmets et al., 1999). ABC7 is an orthologue of yeast Atm1p, which has been shown to play a role in the maturation of cytosolic Fe-S cluster proteins (Kispal et al., 1999). Impairment of Fe-S cluster protein biogenesis may lead to an increase in mitochondrial iron content with similar consequences as for FA (Shoubridge, 2001b).

Animal Models of Mitochondrial Disease

To gain additional insight into the pathogenesis and transmission of mitochondrial disorders, a number of mouse models carrying mutations in either the mitochondrial or nuclear genome have been generated in recent years (Table 1-5). The creation of animal models harbouring mutations in mtDNA has been limited by the fact that it is currently not possible to introduce mutant mtDNA directly into mammalian mitochondria or to

Table 1-5 . Mouse models of mitochondrial disease.

Model	Affected Gene(s)	Phenotype	Reference
MtDNA deletion	6 tRNA genes, 7 structural genes	Premature death (renal failure)	(Inoue et al., 2000)
point mutation in mtDNA	16S rRNA gene	Myopathy, dilated cardiomyopathy, perinatal or embryonic lethality	(Sligh et al., 2000)
Complex I inhibition	None	Optic neuropathy	(Zhang et al., 2002)
Sod2 -/-	Mn superoxide dismutase	Neonatal dilated cardiomyopathy	(Li et al., 1995)
Sod2 -/-	Mn superoxide dismutase	Anemia, neuronal degeneration, motor disturbances	(Lebovitz et al., 1996)
Ant1 -/-	Heart/muscle isoform of the adenine nucleotide translocator	Myopathy, cardiomyopathy	(Graham et al., 1997)
Tfam -/-	Mitochondrial transcription factor A	Embryonic lethality	(Larsson et al., 1998)
Tfam -/- (heart, muscle)	Mitochondrial transcription factor A	Dilated cardiomyopathy, conduction defects	(Wang et al., 1999)
Tfam -/- (pancreatic β-cells)	Mitochondrial transcription factor A	Diabetes	(Silva et al., 2000)
Frda -/-	Frataxin gene	Embryonic lethality	(Cossee et al., 2000)
Frda -/- (striated muscle)	Frataxin gene	Cardiac hypertrophy	(Puccio et al., 2001)
Frda -/- (neurons, cardiac muscle)	Frataxin gene	Cardiac hypertrophy, progressive large neuron dysfunction	(Puccio et al., 2001)
Surf1 -/-	Surf1 gene (COX assembly factor)	Embryonic or perinatal lethality (no neurological symptoms)	(Agostino et al., 2003)
Cox17 -/-	Cox17 gene (COX assembly factor)	Embryonic lethality	(Takahashi et al., 2002b)

introduce isolated mitochondria into mammalian cells (Inoue et al., 2000). Consequently, recent approaches have involved transgenic technology that relies on the creation of cytoplasmic hybrids (or cybrids) to introduce mitochondria carrying mutant mtDNA into mouse ρ^0 cells, which lack mtDNA. In one study, a somatic mouse cell containing a 4696 bp deletion in mtDNA (affecting six tRNA genes and seven structural genes) was fused to murine ρ^0 cells. The resulting cybrids were enucleated to produce cytoplasts, which were electrofused with pronuclear stage embryos and transferred into the oviducts of pseudopregnant females (Inoue et al., 2000). The mutant mtDNA was transmitted to subsequent generations and resulted in mitochondrial dysfunction in a variety of tissues. Mice harbouring predominantly deleted mtDNA died of renal failure at approximately six months of age. Interestingly, humans do not usually show maternal transmission of deleted mtDNA, nor is renal failure a common symptom associated with mitochondrial disease (Shoubridge, 2000). In another very similar investigation, cytoplasts carrying a point mutation in the 16S rRNA gene conferring resistance to chloramphenicol were electrofused to mouse ES cells that had been treated with a mitochondrial toxin and injected into blastocysts (Sligh et al., 2000). Germ-line transmission of the mutant mtDNA resulted in homoplasmic and heteroplasmic mice with a severe phenotype of myopathy, dilated cardiomyopathy, mitochondrial abnormalities, and perinatal or embryonic lethality. More recently, a mouse model for optic neuropathy was created by intravitreal injection of rotenone, a complex I inhibitor (Zhang et al., 2002). The effect of the induced mitochondrial dysfunction is consistent with the loss of retinal ganglion cells in the human disease, LHON.

Murine models of mitochondrial disease resulting from lesions in nuclear genes can be created using classical transgenic experiments. The targeted disruption of six nuclear genes have been reported, including *Sod2* (Li et al., 1995; Lebovitz et al., 1996), *Ant1* (Graham et al., 1997), *Tfam* (Larsson et al., 1998; Wang et al., 1999; Silva et al., 2000), *Frda* (Cossee et al., 2000; Puccio et al., 2001), *Surf1* (Agostino et al., 2003), and *Cox17* (Takahashi et al., 2002b). These experiments have been useful in illustrating several points regarding the use of mice in the generation of models of human mitochondrial disease. To begin, the contrasting phenotypes in the two *Sod2* knock-out mice that have been published indicate the importance of genetic background in the manifestation of mitochondrial disease (Smeitink et al., 2001). In the first *Sod2* -/- mouse, homozygous mutant mice died of a neonatal dilated cardiomyopathy (Li et al., 1995). The second *Sod2* knock-out mouse, which was generated using a different strain of mouse, resulted in a phenotype of severe anemia, neuronal degeneration in the brain, and progressive motor disturbances (Lebovitz et al., 1996). The ablation of the *Surf1* gene in mice demonstrates that the absence of a certain gene product in mice can result in a disparate phenotype in humans. The inactivation of *Surf1* in mice results in high embryonic or neonatal lethality, muscle weakness, poor motor performance, decreased complex IV activity in skeletal muscle, liver, heart, and brain, and skeletal muscle abnormalities (Agostino et al., 2003). The *Surf1* mutant mouse does not demonstrate any brain abnormalities or neurological symptoms, contrasting the phenotype of *SURF-1* mutations in humans, which are primarily associated with Leigh syndrome, a severe early-onset neurodegenerative disorder (Shoubridge, 2001c). While mice lacking Ant1 exhibit features of a classical mitochondrial myopathy and cardiomyopathy, the

phenotype in the *Ant1* knockout mouse is more severe than that which is caused by *ANT1* mutation in humans, which manifests as adPEO (Kaukonen et al., 2000). This suggests that the phenotype resulting from the complete absence of *Ant1* in the knock-out mouse is not an accurate reflection of the milder phenotype in humans, which most likely results from mutated, but partially functional, *ANT1*. A conditional disruption, rather than a complete ablation, of a given gene may provide a better model of disease in some cases. While the complete absence of *Frda* in all tissues results in embryonic lethality (Cossee et al., 2000), the conditional inactivation of *Frda* in striated muscle, neurons, and cardiac muscle together result in a phenotype that is consistent with many of the clinical features seen in FA (Puccio et al., 2001). The complete absence of several of the nuclear genes, such as *Tfam*, *Frda*, and *Cox17*, results in embryonic lethality, suggesting that these genes play an essential role in embryonic development (Larsson et al., 1998; Cossee et al., 2000; Takahashi et al., 2002b).

Cytochrome Oxidase Deficiency in Humans

Cytochrome oxidase deficiencies are a clinically and genetically heterogeneous group of disorders stemming from defects in the function of the terminal electron acceptor of the mitochondrial respiratory chain. Although mutations have been found in the three subunits genes encoded by the mitochondrial genome (Table 1-6), no defects have been identified to date in the nuclear genes encoding the remaining ten subunits (Barrientos et al., 2002a). The recent identification of mutations in a number of human genes involved in COX biogenesis (Table 1-7) suggest that lesions in COX assembly factors are the most common cause of isolated COX deficiency (Shoubridge, 2001a). This is in agreement with earlier biochemical studies, which proposed that defects in

Table 1-6. Isolated cytochrome oxidase deficiencies associated with mutations in mitochondrial DNA-encoded cytochrome oxidase subunit genes.

Gene	Mutation	Phenotype	Reference
COX I	Missense mutation	Acquired sideroblastic anemia	(Gattermann et al., 1997)
COX1	Missense mutation	Acquired sideroblastic anemia	(Gattermann et al., 1997)
COX I	5 bp deletion	Motor neuron disease	(Comi et al., 1998)
COX I	Nonsense mutation	Encephalomyopathy	(Bruno et al., 1999)
COX I	Nonsense mutation	Recurrent myoglobinuria	(Karadimas et al., 2000)
COX II	Missense mutation	Encephalomyopathy	(Clark et al., 1999)
COX II	Missense mutation	Proximal limb weakness	(Rahman et al., 1999)
COX III	Missense mutation[a]	MELAS	(Manfredi et al., 1995)
COX III	15 bp deletion	Myopathy, myoglobinuria	(Keightley et al., 1996)
COX III	Nonsense mutation	Exercise intolerance, encephalomyopathy	(Hanna et al., 1998)
COX III	1 bp insertion	Leigh-like syndrome	(Tiranti et al., 2000)

[a] first report of a mutation in a mitochondrial DNA-encoded cytochrome oxidase subunit

Table 1-7. Human cytochrome oxidase assembly factor genes associated with isolated cytochrome oxidase deficiency.

Yeast Gene	Human Gene	Gene Function	Phenotype	Reference
COX10	*COX10*	Farnesylation of protoheme	Leukodystrophy and tubulopathy	(Valnot et al., 2000b)
COX15	*COX15*	Hydroxylation of heme O	Fatal infantile hypertrophic cardiomyopathy	(Antonicka et al., 2003)
SCO1	*SCO1*	Copper provision	Neonatal encephalopathy and hepatic failure	(Valnot et al., 2000a)
	SCO2	Copper provision	Fatal infantile cardioencephalomyopathy	(Papadopoulou et al., 1999)
SHY1	*SURF1*	unknown	Leigh syndrome	(Tiranti et al., 1998; Zhu et al., 1998)

COX assembly were a major underlying cause of COX deficiency in humans (Glerum et al., 1988; Lombes et al., 1991)

Clinical characterization of patients with COX deficiency suggest that there are five distinct phenotypes found in humans (Robinson, 2000). The most common clinical manifestation of COX deficiency is Leigh syndrome, which results in progressive deterioration of the basal ganglia and brain stem. A second Leigh syndrome phenotype (LSFC), which is less severe than the classical form, occurs only in the Charlevoix and Saguenay-Lac-St. Jean region of Québec. Affected individuals have severely reduced COX activity in liver and brain, with 50% wild-type activity in skeletal muscle and near normal levels in kidney and heart. A genome-wide association study was used to map the LSFC gene to chromosome 2p16-21 (Lee et al., 2001). An integrative genomics approach, involving mRNA neighbourhood analysis and organelle proteomics, led to the identification of *LRPPRC*, a gene in the LSFC candidate region that was mutated in affected individuals (Mootha et al., 2003). While the function of LRPPRC (leucine-rich pentatricopeptide repeat-containing protein) is unknown, the protein shares weak homology with Pet309p, suggesting that LRPPRC may be involved in the binding of mtDNA-encoded COX mRNA. Fatal infantile COX deficiency, which results in severe COX deficiency in kidney and muscle, presents with renal Fanconi syndrome and myopathy. The two final phenotypes seen in human COX deficiency include myopathy with cardiomyopathy, and tubulopathy, leukodystrophy, and ataxia (Robinson, 2000).

Expression analysis of human COX assembly factors indicates that they are all encoded by ubiquitously expressed housekeeping genes and are expressed to varying levels in the different tissues (Shoubridge, 2001a). Currently, virtually nothing is known

about the regulation of COX assembly factor gene expression in different tissues. It is also unclear why some tissues in individuals with COX deficiency show relatively modest reductions in enzyme activity and no apparent assembly defect, while other tissues are severely affected (Shoubridge, 2001a).

The molecular basis of COX deficiency in the majority of patients remains unknown. Recent identification of human COX assembly genes using a candidate gene approach, based on homologous genes found in model organisms such as yeast, and functional complementation, using techniques such as microcell-mediated chromosome transfer, suggest that these methodologies will continue to be useful for COX assembly factor gene identification in the future (Shoubridge, 2001a).

The data presented in this thesis add to the characterization of both the human and yeast *COX17* genes. The gene structure, expression, and chromosomal localization of human *COX17* and a pseudogene, *COX17P*, will be described. The deciphering of the *COX17* genomic structure has allowed this gene to be assessed for mutations in COX deficient patients. In addition, the subcellular localization of human COX17 will be demonstrated. Although a mitochondrial localization for mammalian COX17 was excluded in a previous report, it will be shown that human COX17 has a similar cellular distribution to yeast Cox17p and can be found in the mitochondrion. Mutational analysis of yeast *COX17* will also be discussed. The results of the mutagenesis study suggest that Cox17p is specifically involved in the provision of copper to the Cu_A site on COX II, acting upstream of Sco1p. Together, all of the experiments described in this thesis contribute to

the understanding of the structure and function of *COX17*, a candidate gene in human COX deficiency.

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Chapter 2. Materials and Methods

DNA CLONES AND CONSTRUCTS

Isolation of P1 18110 DNA

Using the human COX17 cDNA, a P1 clone (P1 18110) harbouring human *COX17* was isolated by Genome Systems (St. Louis, USA). P1 18110 DNA was isolated according to the instruction manual provided by Genome Systems. At the end of the DNA isolation, any contamination was removed by spot dialysis for 30 minutes on a filter floating on a bath of sterile water.

Isolation of BAC DNA

Bacterial artificial chromosomes (BACs) containing human *COX17* (BAC 274K10, BAC 26I10, and BAC 59M4) and *COX17P* (BAC 271E16, BAC 162M22, and BAC 271I16) were isolated by the Medical Research Council (MRC) Genome Resource Facility at the Hospital for Sick Children, Toronto, Canada, using the human *COX17* cDNA as a probe. BAC DNA used for Southern blotting was isolated according to the protocol provided by the MRC Genome Resource Facility. BAC DNA used in sequencing reactions was isolated with the Qiagen Plasmid Midi Kit according to the manufacturer's instructions.

Construct: pCOX17H/ST2

The human *COX17* pseudogene, *COX17P*, was amplified from human genomic DNA using primers COX17H-7 and COX17-4, and ligated into the pGEM T-Easy vector (Promega). The orientation of the insert was verified by restriction enzyme digest and DNA sequencing (M13 forward and reverse primers) using the LongReadIR automated sequencing system (LiCor). A double restriction enzyme digest with *Sph*I and *Sac*I was performed to excise the insert from the vector. *COX17P* was ligated into *Sph*I/*Sac*I-

digested pMGL4 (D.M. Glerum and D.L. Adams, unpublished results) to generate the plasmid, pCOX17/ST2. Fragments cloned into pMGL4, a multicopy shuttle vector with a YEp352 backbone, are under the control of the *S. cerevisiae ADH1* promoter and terminator.

Construct: pMGL4-hCOX17

The human *COX17* cDNA was amplified from pG74H/T1 (Amaravadi et al., 1997) using primers COX17H-7 and COX17H-4 and cloned into the pGEM T-Easy vector (Promega) to generate pGEM-hCOX17. The plasmid, pGEM-hCOX17, was linearized with *Sph*I and treated with Klenow reagent to create blunt ends. The linearized vector was then digested with *Sac*I to excise the *COX17* cDNA fragment from the plasmid and ligated into the multiple cloning site of pMGL4, which had been digested with *Sac*I and *Sma*I, to generate pMGL4-hCOX17.

Construct: pCDNA3.1-hCOX17NFL and pCDNA3.1-hCOX17CFL

To create pCDNA3.1-hCOX17NFL, the human *COX17* cDNA was amplified from pG74H/T1 (Amaravadi et al., 1997) using primers 17HFLAGN-F (5'-gacgacgatatcaccatgggtggtgccgactacaaggacgatgacgataagatgccgggtctggttgactc-3') and 17HFLAG-R (5'- gacgacctcgagccatttcatattttaaatcctag-3'). This primer pair was used to introduce an *Eco*RV site upstream of the *COX17* coding region, an in-frame FLAG epitope at the 5' end of the *COX17* coding region, and *Xho*I site downstream of the *COX17* coding region. The *COX17* cDNA PCR fragment containing the added restriction sites and FLAG epitope was electrophoresed on a 1% agarose gel, excised from the gel, and purified using the QIAEX II Gel Extraction Kit (Qiagen). The PCR fragment was then digested with *Eco*RV and *Xho*I, purified with glass milk, and ligated

into the pCDNA3.1/hygro+ vector (Invitrogen Life Technologies; generously provided Dr. Alan Underhill, Department of Medical Genetics, University of Alberta). The resulting plasmid, pCDNA3.1-hCOX17NFL, was verified by DNA sequencing using the LongReadIR automated sequencing system (LiCor).

The plasmid, pCDNA3.1-hCOX17CFL, was created in a similar manner as pCDNA3.1-hCOX17NFL, except that primers 17HFLAGC-F (5'-gacgacgatatcaccatgccgggtctggttgactc-3') and 17HFLAGC-R (5'-gacgacctcgagtcaggcaccacccttatcgtcatcgtccttgtagtctattttaaatcctagggctct-3') were used to introduce an *Eco*RV site upstream of the *COX17* coding region, an in-frame FLAG epitope at the 3' end of the *COX17* coding region, and *Xho*I site downstream of the *COX17* coding region.

Constructs: pCOX17-571NFLAG and pCOX17-571CFLAG

The plasmid, pCOX17-571NFLAG, which contains DNA sequence encoding the FLAG epitope fused in-frame to the 5' end of the yeast G41* *cox17* mutant, was created using the Quikchange Site-Directed Mutagenesis Kit (Stratagene). The mutagenesis reaction was carried out using 10 ng of pCOX17/ST571 DNA and primers 571NFL-F (5'-ctacacaatcaattatacccaatgggtggtgctgactacaaggacgatgacgataagatgactgaaactgacaagaaac-3') and 571NFL-R (5'- gtttcttgtcagtttcagtcatcttatcgtcatcgtccttgtagtcgg-caccacccattgggtataattgattgtgtag-3'). A total of 18 cycles were used during the PCR amplification step of the mutagenesis reaction. The construct was verified by DNA sequencing using the LongReadIR automated sequencing system (LiCor).

To create plasmid, pCOX17-571CFLAG, which contains DNA sequence encoding the FLAG epitope fused in-frame to the 3' end of the yeast *cox17* G41* mutant,

cox17 harbouring the G41* mutation was amplified from pCOX17/ST571 using primers 17RM233-N (5'-cagtgccaagcttgtaaccac-3') and 17RM571-CFLAG (5'-gacgacgcatgcctaagcaccactttgtcatcgtcatctttgtaatcattgaataagatgcatgtatc-3'). The PCR fragment was digested with *Sph*I and *Hin*dIII and ligated into similarly digested YEp352. The construct was verified by DNA sequencing using the LongReadIR automated sequencing system (LiCor). The PCR fragment was then subcloned into YCplac33, which had been digestd with *Sph*I and *Hin*dIII.

Construct: pCOX17-233NFLAG

To create pCOX17-233NFLAG, which contains DNA sequence encoding the FLAG epitope fused in-frame to the 5' end of the yeast *cox17* K59* mutant, the Quikchange Site-Directed Mutagenesis Kit (Stratagene) was used. The mutagenesis reaction was carried out using 10 ng of pCOX17/ST233 DNA and primers 571NFL-F (5'-ctacacaatcaattatacccaatgggtggtgctgactacaaggacgatgacgataagatgactgaaactgacaagaaac-3') and 571NFL-R (5'- gtttcttgtcagtttcagtcatcttatcgtcatcgtccttgtagtcggcac-cacccattgggtataatt gattgtgtag-3'). A total of 18 cycles were used during the PCR amplification step of the mutagenesis reaction. The construct was verified by DNA sequencing using the LongReadIR automated sequencing system (LiCor). The *COX17* fragment was excised with *Sph*I and *Hin*dIII and ligated into similarly digested YCplac33.

Construct: pCOX17-233CXpress

Plasmid pCOX17-233CXpress, which contains DNA sequence encoding the Xpress epitope (Invitrogen Technologies) fused in-frame to the 3' end of the yeast *cox17* K59* mutant, was created by amplifying *cox17* from pCOX17/ST233 using primers 17RM233-

N (5'-cagtgccaagcttgtaaccac-3') and 17RM233-C (5'-gtcgtcgcatgcctacttatcgtcatcgtcgtac-agatccatgcactctttgtacttttc-3'). The PCR fragment was digested with *Sph*I and *Hin*dIII and ligated into similarly digested YEp352. The construct was verified by DNA sequencing using the LongReadIR automated sequencing system (LiCor). The PCR fragment was then subcloned into YCplac33, which had been digested with *Sph*I and *Hin*dIII.

Constructs: pCOX17-NFLAG and pCOX17-CFLAG

The plasmid, pCOX17-NFLAG, which contains DNA sequence encoding the FLAG epitope fused in-frame to the 5' end of yeast *COX17*, was created using the Quikchange Site-Directed Mutagenesis Kit (Stratagene). The mutagenesis reaction was carried out using 10 ng of pG74/ST8 DNA (Glerum et al., 1996) and primers 571NFL-F (5'-ctacacaatcaattatacccaatgggtggtgctgactacaaggacgatgacgataagatgactgaaactgacaagaaac-3') and 571NFL-R (5'- gtttcttgtcagtttcagtcatcttatcgtcatcgtccttgtag-tcggcaccacccattgggtataatt gattgtgtag-3'). A total of 18 cycles were used during the PCR amplification step of the mutagenesis reaction. The construct was verified by DNA sequencing using the LongReadIR automated sequencing system (LiCor).

MOLECULAR BIOLOGY TECHNIQUES

Southern Blotting – Nylon Membrane

Five µg of human genomic DNA (anonymous DNA samples generously provided by Dr. Martin Somerville, Molecular Diagnostics Laboratory, University of Alberta), or BAC DNA were digested overnight with the appropriate restriction enzyme (*Eco*RI, *Hind*III, or both) and electrophoresed in a 0.8% agarose gel, made with 1X TBE, at 35V for 17 hours. After electrophoresis, the gel was stained in ethidium bromide (0.5 mg/mL in 1X TBE) for 20 to 30 minutes. The gel was then rinsed in water, incubated in 0.25 M

HCl for 30 minutes, followed by denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 40 minutes with rocking. After rinsing with water, the gel was neutralized in 1.5 M NaCl/0.5 M Tris-Cl for 40 minutes with rocking. The DNA was transferred overnight to Hybond-N nylon membrane (Amersham Life Science) using 20X SSC. After transfer, the DNA was bound to the membrane by UV cross-linking or by baking at 80°C for two hours.

The membrane was pre-hybridized in Church and Gilbert hybridization solution (Church and Gilbert, 1984) (0.34 M Na_2HPO_4, 0.16 M NaH_2PO_4, 7% SDS, 1% BSA) for one hour at 65°C. The hybridization solution was replaced with fresh solution containing $1x10^6$ cpm/mL of [α-^{32}P]dCTP-labelled *COX17* cDNA and 50 µg/mL salmon sperm carrier DNA (both denatured in a boiling water bath for five minutes and placed on ice before adding to the hybridization solution). The Southern blot was probed overnight at 65°C. After hybridization, the membrane was washed twice for 15 minutes in 2X SSC/0.1% SDS, twice for 15 minutes in 0.2X SSC/0.1% SDS, and twice for 15 minutes in 0.1X SSC/0.1% SDS at 55°-65° C. The blot was wrapped in Saran Wrap and exposed to Kodak X-OMAT film overnight at –80°C.

Southern Blotting – Nitrocellulose Membrane

Two µg of DNA (P1 DNA) were digested with the appropriate restriction enzyme (*Eco*RI, *Hind*III, or both) and electrophoresed in a 0.8% agarose gel made with 1X TBE at 35 V for 17 hours. After electrophoresis, the gel was stained in ethidium bromide (0.5 mg/mL in 1X TBE) for 20 to 30 minutes. After rinsing with water, the gel was soaked in denaturing solution (0.4 M NaOH, 1.5 M NaCl) for twenty minutes with gentle rocking, followed by neutralization solution (1M Tris-Cl pH 8.0, 3M NaCl) for 10 minutes with

rocking. The DNA was transferred overnight to Trans-Blot Transfer Medium (BIO-RAD), using 20X SSC. After transfer, the membrane was rinsed with 2X SSC for 5 minutes and blotted dry with Whatman paper. The DNA was bound to the membrane by baking at 80°C for two hours.

For hybridization, the nitrocellulose membrane was wetted with 6X SSC and probed overnight at 65°C in hybridization solution (6X SSC, 1% Sarkosyl) containing $1x10^6$ cpm/mL of [α-^{32}P]dCTP-labelled *COX17* cDNA and 50 µg/mL salmon sperm carrier DNA (both denatured in a boiling water bath for five minutes and placed on ice before adding to the hybridization solution). After hybridization, the membrane was washed twice for 15 minutes in 2X SSC/0.1% SDS and twice for five minutes in 5 mM Tris, pH 8.0. The blot was wrapped in Saran Wrap and exposed to Kodak X-OMAT film overnight at –80°C.

Radioactive Labelling of DNA Probes for Southern and Northern Blot Hybridization

Five to 10 µL of DNA were labeled with [α-^{32}P]dCTP (approximately 50 µCi) according to the instructions for the Random Primers DNA Labeling System (GibcoBRL, Life Technologies). After one hour at room temperature, the reaction was purified on a Sephadex G-50 Nick Column (Pharmacia), according to the manufacturer's instructions. After purification, one µL of the probe was counted using a scintillation counter.

Hybridization of a cDNA Probes to a Multiple Tissue Northern Blot

A human adult Multiple Tissue Northern (MTN) blot was purchased from Clontech Laboratories and hybridized with human *COX17* (isolated from pG74H/T1 (Amaravadi et al., 1997)), *GAPDH*, and *S26* cDNAs. Both the *GAPDH* and *S26* cDNAs

were isolated from plasmids generously provided by John Forbes (the lab of Dr. Diane COX, Department of Medical Genetics, University of Alberta). The MTN blot was incubated for one hour in ExpressHyb solution (Clontech Laboratories) at 68°C prior to the addition of the radioactive probe. Salmon sperm carrier DNA and [α-^{32}P]dCTP radioactively-labelled probe (either human *COX17*, *GAPDH*, and *S26* cDNAs) were denatured in a boiling water bath for five minutes and added to 5 mL of fresh ExpressHyb at a concentration of 0.02 mg/mL and $1x10^6$ cpm/mL, respectively. The prehybridization buffer was replaced with the fresh buffer containing the probe and carrier DNA and incubated for one hour at 68°C. The MTN blot was rinsed several times in 2X SSC/0.05% SDS and then washed for 45 minutes in the same solution (the solution was changed three times during the wash). The blot was washed twice for 20 minutes in 0.1X SSC/0.1% SDS at 50°C. After the final wash, the blot was immediately wrapped in Saran Wrap and exposed to X-OMAT film (Kodak). The film was developed after a 24 hour exposure.

Expression Analysis of *COX17* and *COX17P* in lymphoblastoid RNA

Total RNA, which was kindly provided by James Friedman (the lab of Dr. Michael Walter, Departments of Ophthalmology and Medical Genetics, University of Alberta), was isolated from a lymphoblastoid cell line using TRIzol reagent (GibcoBRL, Life Technologies). First-strand cDNA synthesis was carried out with two µg RNA and an oligo$(dT)_{12\text{-}18}$ primer (GibcoBRL, Life Technologies), followed by PCR of the reverse transcribed products. The forward primer, COX17H-1 (5'-ggaagtgactgcagacgaatcgg-3'), which is specific for the 5' end of *COX17*, was used with the reverse primer, COX17H-4 (5'-agttcgtcaaagaactccca-3'), to detect the expression of *COX17*. Similarly, primer

COX17B-2 (5'ttaaagaaatcggacgaattgg-3'), which is specific for the 5' end of *COX17P*, was used with COX17H-4 to test for the expression of *COX17P*.

Radiation Hybrid Mapping of *COX17* and *COX17P*

Radiation hybrid mapping was carried out using the GeneBridge 4 Human/Hamster Radiation Hybrid Panel (Research Genetics), which is composed of 93 radiation hybrids containing fragments of the whole human genome in a hamster background (kindly provided by Dr. Michael Walter, Departments of Ophthalmology and Medical Genetics, University of Alberta). Primers COX17H-3 (5'-tgagtctcaggagaagaagc-3') and COX17H-4 were used to map *COX17P*. *COX17* was localized using intronic primers, 18110-13 (5'-gccacttcctgtggaaag-3') and 18110-18 (5'-gacttgaagagttagctac-3').

Direct Sequencing of *COX17* and *COX17P*

COX17 was sequenced manually from P1 18110 and BAC 274K10 DNA. *COX17P* was sequenced from BAC 271E16 and from a PCR fragment amplified from human genomic DNA using primers COX17H-7 (5'-aggctggcatagatttggc-3') and COX17H-4. Sequencing reactions were carried out using the Thermo-Sequenase Radiolabeled Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech). Sequencing reactions were electrophoresed in 6% polyacrylamide gels using glycerol tolerant buffer.

Purification of DNA Fragments with Glass Milk

DNA samples were electrophoresed in 1% agarose gels. The fragment of interest was excised from the gel, placed in a 1.5 mL microcentrifuge tube, and weighed. Three volumes of sodium iodide solution were added to the DNA fragment, which was

incubated at 65°C until the agarose had melted. After adding 15 µL of glass milk (made with crushed silica) to the agarose suspension, the tube was incubated at room temperature for 10 minutes. The tube was mixed by vortexing every few minutes during the incubation. The tube was centrifuged in a microcentrifuge for 30 seconds at 7000 rpm to pellet the glass milk. The pellet was washed three times with 700 µL cold NEET solution (100 mM NaCl, 1 mM EDTA, 10 mM Tris, pH 7.5, 50% ethanol). Fifteen µL of water was added to the glass milk pellet, which was mixed by vortexing and incubated for 10 minutes at 65 °C. The tube was centrifuged at maximum speed for 1 minute and the supernatant transferred to a fresh tube. The elution of DNA was repeated by adding another 15 µL of water to the glass milk pellet. The eluates were pooled, yielding a final volume of 30 µL. The recovery of the DNA fragment was quantitated on a 1% agarose gel.

Yeast Transformation by Lithium Acetate (LiAc) Method

The following protocol is a modification of the method described by Schiestl and Gietz (Schiestl and Gietz, 1989). The strain to be transformed was inoculated into 10 mL of YPD liquid medium and grown overnight at 30°C with shaking. Two mL of the overnight culture was used to inoculate 100 mL of YPD, which was grown for four hours at 30°C with shaking (OD_{600} should be between 0.6 and 1.0). The culture was centrifuged at 3000 rpm in two sterile 50 mL conical tubes for 5 minutes at room temperature. The supernatant was poured off and the pellets were resuspended in 10 mL TEL (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 M LiAc), combined, and centrifuged at 3000 rpm for 5 minutes. After the supernatant was discarded, the pellet was resuspended in 0.9 mL of TEL. The cells were placed in sterile 1.5 mL microcentrifuge tubes in 100 µL aliquots.

To each aliquot, 5 µL of transforming DNA and 5 µL salmon sperm carrier DNA (10 mg/mL; denatured at 90°C for 10 minutes) were added. The cells and DNA were mixed and incubated at room temperature for 30 minutes. Cells were then incubated for 45 minutes at room temperature after adding 0.7 mL PEG/TEL buffer (40% polyethylene glycol, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 M LiAc) and mixing well. The cells were heat shocked at 45°C for 10 to 15 minutes and centrifuged in a microcentrifuge for 10 seconds. After removing the supernatant, the cell pellets were resuspended in 0.2 mL TE (pH 8.0) and centrifuged again for 10 seconds. After discarding the supernatant, the cells were resuspended in 50 to 100 µL TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) and spread on selective minimal medium (WO). Yeast colonies were apparent on plates after three days incubation at 30°C.

Random Mutagenesis of Yeast *COX17*

To introduce random mutations into the yeast *COX17* gene, a two-step PCR-based protocol was used, based on the method described by Xu *et al.* (Xu et al., 1999). In the first PCR (total volume of 50 µL), primers yCOX17-3 (5'-ttataagcttgtaaccacctaac-3') and yCOX17-4 (5'-gatagtgcatgcagtagaag-3') were used to amplify yeast *COX17* from pG74/ST8, a vector containing the gene cloned into a *Hin*dIII site (Glerum et al., 1996), in the presence of the mutagen, Mn^{2+}. The yCOX17-4 primer was designed to introduce an *Sph*I site downstream of the *COX17* coding region. Three different concentrations of Mn^{2+} were tried in the first PCR, including 40 µM, 80 µM, and 160 µM. In the second PCR (total volume of 50 µL), primers yCOX17-3 and yCOX17-4 were used to amplify *COX17* in the presence of the mutagen, dITP (Amersham Pharmacia Biotech), with 2 µL of the first PCR serving as the template DNA. Three different concentrations of dITP

were tried in the second PCR, including 40 μM, 80 μM, and 160 μM. The PCR fragment amplified in the second PCR was electrophoresed in a 1% agarose gel and purified using the QIAEX II Gel Extraction Kit (Qiagen). Following a *Hin*dIII/*Sph*1 double digest, the PCR fragment was purified with the QIAquick PCR Purification Kit (Qiagen). The PCR fragment was ligated into similarly digested YEp352 and transformed into DH5α *Escherichia coli*. Plasmid DNA from individual bacterial colonies was isolated by the Triton plasmid DNA preparation method and sequenced using the LongReadIR automated sequencing system (LiCor). Plasmids containing a mutation in the *COX17* coding region were digested with *Hin*dIII and *Sph*1. The excised *COX17* fragment was ligated into the CEN plasmid, YCplac33 (Gietz and Sugino, 1988) (kindly provided by Dr. Bernard Lemire, Department of Biochemistry, University of Alberta). Plasmids harbouring mutagenized *COX17* were transformed into the *cox17* null mutant strain, plated on WO medium containing histidine, adenine, and leucine, and replica-plated to EG medium to assess respiratory growth.

Site-Directed Mutagensis of Yeast *COX17*

Specific mutations were introduced into yeast *COX17* using the Quikchange Site-Directed Mutagenesis Kit (Stratagene). Site-directed mutagenesis reactions were carried out according to the manufacturer's manual, using 10 ng of pG74H/ST8 (Glerum et al., 1996) as template DNA. The primers used in site-directed mutagenesis reactions are listed in Table 2-1. Plasmids carrying mutations were isolated by the Triton plasmid DNA preparation method and sequenced using the LongReadIR automated sequencing system (LiCor). *COX17* fragments harbouring mutations were excised from pG74/ST8 with a *Hin*dIII digest and ligated into YEp352 and YCplac33. The resulting plasmids

Table 2-1. Primer pairs used in the site-directed mutagenesis of yeast *COX17*.

Mutation	Forward Primer	Reverse Primer
K21A	COX17K21A-F 5'-gaggacaaacctgcgccatgttgcgtttg-3'	COX17K21A-F 5'-caaacgcaacatggcgcaggtttgtcctc-3'
P22A	17P22A-F 5'-ggacaaacctaaggcatgttgcgtttgtaagcc-3'	17P22A-R 5'-ggcttacaaacgcaacatgccttaggtttgtcc-3'
V25T	17V525T-F 5'-cctaagccatgttgcacttgtaagccagaaaaggagg-3'	17V525T-R 5'-cctccttttctggcttacaagtgcaacatggcttagg-3'
K30A	COX17K30A-F 5'-gtaagccagaagcggaggagcgggatac-3'	COX17K30A-R 5'-gtatcccgctcctccgcttctggcttac-3'
R33A	17R33A-F 5'-gccagaaaaggaggaggcggatacatgcatc-3'	17R33A-R 5'-gatgcatgtatccgcctcctcctttctggc-3'
R33D	17R33D-F 5'-ccagaaaaggaggaggatgatacatgcatcttattc-3'	17R33D-R 5'-gaataagatgcatgtatcatcctcctcctttctgg-3'
D34A	17D34A-F 5'-gaaaaggaggagcgggctacatgcatcttattc-3'	17D34A-R 5'-gaataagatgcatgtagcccgctcctcctttc-3'
D34R	17D34R-F 5'-cagaaaaggaggagcggcggacatgcatcttattc-3'	17D34R-R 5'-gaataagatgcatgtccgccgctcctcctttctg-3'
F39S	17F39S-F 5'-gcgggatacatgcatcttatccaatggacaagac-3'	17F39S-R 5'-gtcttgtccattggataagatgcatgtatcccgc-3'
E45A	COX17E45A-F 5'-ggacaagactctgcaaaatgcaaggaattc-3'	COX17E45A-R 5'-gaattccttgcattttgcagagtcttgtcc-3'
E52A	COX17E52A-F 5'-caaggaattcattgcaaagtacaaagagtg-3'	COX17E52A-R 5'-cactctttgtactttgcaatgaattccttg-3'
K55A	COX17K55A-F 5'-cattgaaaagtacgcagagtgcatgaaggg-3'	COX17K55A-R 5'-cccttcatgcactctgcgtacttttcaatg-3'
E56A	COX17E56A-F 5'-cattgaaaagtacaaagcgtgcatgaaggg-3'	COX17E56A-R 5'-cccttcatgcacgctttgtacttttcaatg-3'
M58A	17M58A-F 5'-gtacaaagagtgcgcgaagggttatggcttcg-3'	17M58A-R 5'-cgaagccataacccttcgcgcactctttgtac-3'

were transformed into the *cox17* null mutant strain, plated on WO medium containing histidine, adenine, and leucine, and replica-plated to EG medium to assess respiratory growth.

Triton Plasmid DNA Preparation

A bacterial strain carrying the plasmid of interest was streaked over the entire surface of an LB plate and incubated at 37°C overnight. The cells were scraped from the confluent plate with a sterile spatula, placed in a 15 mL conical tube, and resuspended in one mL of cold buffer containing 5% sucrose and 50 mM Tris, pH 8.0. After adding one mL of lysozyme buffer (4.5 mg/mL lysozyme, 22.7 mM Tris pH 8.0, 113.6 mM EDTA, 0.9 mg/mL RNase A), the tube was mixed by inversion, and incubated on ice for 30 minutes. One mL of cold Triton X-100 lysis buffer (0.3% Triton X-100, 0.185 M EDTA, 0.15 M Tris pH 8.0) was added and the contents of the tube were mixed by inversion. The cell mixture was transferred to a polyallomer thick wall ultracentrifuge tube (13 X 56 mm; Beckman) and centrifuged at 40 000 rpm for 20 minutes at 4°C in an Optima TLX ultracentrifuge (Beckman). The supernatant was transferred to a new 15 mL conical tube, to which an equal volume of water-saturated phenol was added. The contents of the tube were mixed by vortexing and centrifuged at 4200 rpm for 5 minutes at 4°C in a J6-MI centrifuge (Beckman). The upper aqueous phase was removed with a Pasteur pipette and placed in a fresh 15 mL conical tube. The tube was filled with ether and mixed by shaking. After the two phases had settled out, the top ether layer was removed with an aspirator. The wash step was repeated twice more or until the lower phase was clear. Once clear, 0.05 volumes of 5M NaCl and three volumes of 100% ethanol were added to the lower phase and mixed by inversion. The tube was centrifuged at 4000 rpm for 10

minutes at 4°C, yielding a small oily pellet in the bottom of the tube. The pellet was dissolved in two mL of 2M ammonium acetate and three volumes of 100% ethanol were added to the tube, which was centrifuged at 4000 rpm for 10 minutes at 4°C. The white DNA pellet was dissolved in 0.3 mL of 2M ammonium acetate and transferred to a 1.5 mL microcentrifuge tube. After adding 2.5 mL of ethanol, the tube was centrifuged at maximum speed in a microcentrifuge. The DNA pellet was washed with 80% ethanol/0.2 mM EDTA, followed by a second wash with 80% ethanol. The DNA was dried in the SpeedVac Plus (Savant) for approximately 10 minutes and resuspended in 50 to 100 µL of water. The concentration of the plasmid DNA was determined using the dna95 software on a Shimadzu UV-Visible UV-1601 PC spectrophotometer.

***E. coli* Plasmid Miniprep**

Individual bacterial colonies were streaked in small patches (approximately 1 cm^2) on LB medium and incubated overnight at 37°C. Cells were collected from the plates using sterile toothpicks and placed in 1.5 mL microcentrifuge tubes containing 100 µL of cold lysis buffer (50 mM glucose, 25 mM Tris-Cl pH 8.0, 10 mM EDTA, 5 mg/mL lysozyme, 0.02 mg/mL RNase A). The tubes were placed on ice for one minute after resuspending the cells in the lysis buffer. Two hundred µL of 0.2 M NaOH/1% SDS were added and the tubes were mixed by inversion. After adding 150 µL of 7.5 M ammonium acetate, the tubes were mixed by shaking and centrifuged for three minutes in a microcentrifuge at maximum speed. The supernatant was decanted to a fresh tube, into which 300 µL of isopropanol containing 0.2% Triton X-100 was added. The tubes were mixed by shaking and centrifuged for 5 minutes at maximum speed. The plasmid DNA was washed with 80 % ethanol/0.2 mM EDTA, followed by a second wash with 80%

ethanol. DNA pellets were dried in the SpeedVac Plus (Savant) and resuspended in 15 to 50 µL of water.

Small-scale plasmid DNA isolation was also carried out using the GenElute Plasmid Miniprep Kit (Sigma) according to the protocol provided by the manufacturer.

Transformation of Competent *E. coli* Cells

E. coli cells were made competent according to the method described by Inoue *et al.* (Inoue et al., 1990) and stored in aliquots at –80°C. After thawing competent cells at room temperature, 200 µL aliquots were placed in sterile 1.5 mL microcentrifuge tubes on ice. One to five µL of plasmid DNA were added to the competent cells, which were then incubated on ice for 30 minutes. The cells were heat-shocked at 42°C for 30 seconds and placed on ice for two minutes. Following the addition of 0.8 mL of liquid LB medium, the cell mixtures were transferred to sterile glass tubes (15 X 125 mm; Pyrex) and incubated at 37°C for one hour with shaking (225 rpm). The cells were plated on selective LB medium and incubated at 37°C overnight.

Western Blotting

Protein extracts were prepared in sample buffer (4X stock: 0.19 M Tris-HCl pH 6.8, 3.85% SDS, 38.5% glycerol, 0.02-0.05% bromophenol blue) and separated in 12% or 15% (with glycerol) SDS-PAGE gels prepared according to the Laemmli recipes (Laemmli, 1970). After electrophoresis, proteins were transferred to nitrocellulose (Trans-Blot Transfer Medium; BIO-RAD) using the Mini-PROTEAN II system (BIO-RAD). The transfer was carried out at 100 V for 30 minutes in cold transfer buffer consisting of 200 mM glycine, 25 mM Tris, and 20% methanol. After the transfer, the nitrocellulose membrane was stained with Ponceau S dye solution (2% Ponceau S

[Sigma], 30% trichloroacetic acid, and 30% sulfosalicylic acid), rinsed with water, and blocked for at least 30 minutes in blocking buffer (1.5-5% powdered milk in rinse buffer) with rocking. The blot was placed in fresh blocking buffer containing primary antibody and incubated for one to two hours with rocking. Following a quick rinse in rinse buffer (10 m Tris-HCl pH 8.0, 1 mM EDTA, 150 mM NaCl, 0.1% Triton X-100), the blot was washed three times for 10 minutes in rinse buffer with rocking. The blot was placed in fresh blocking buffer containing secondary antibody conjugated to horseradish peroxidase and incubated for 45 minutes to one hour with rocking. After a quick rinse in rinse buffer + salt (10 m Tris-HCl pH 8.0, 1 mM EDTA, 1 M NaCl, 0.1% Triton X-100), the blot was washed three times for 10 minutes in the same buffer with rocking. Proteins were detected using the ECL Western Blotting Detection Reagents (Amersham Biosciences). The Western blot was wrapped in Saran Wrap and exposed to Kodak X-OMAT or BioMax film, which was developed using a M35A X-OMAT processor (Kodak).

Tricine-SDS-Polyacrylamide Gel Electrophoresis for the Separation of Low Molecular Weight Proteins

The 16.5% polyacrylamide gel system used to separate low molecular weight proteins (in the range of 5-20 kDa) consisted of a lower separation gel, a middle spacer gel, and an upper stacking gel. The protocol is based on the method described by Schagger *et al* (Schagger and von Jagow, 1987). To prepare the separation gel, 5 mL of acrylamide stock B (46.5% acrylamide, 3% bis-acrylamide), 5 mL of gel buffer (3 M Tris-HCl pH 8.45, 0.3% SDS), and 5.4 g of urea were mixed with water to a final volume of 15 mL. The separation gel was polymerized with 50 µL 10% ammonium persulfate

(APS; GibcoBRL) and 7.5 μL TEMED (GibcoBRL). After pouring the separation gel, it was overlayed with water. Once the separation gel had set, the water was removed and the spacer gel was poured so that it was one-sixth of the volume of the separation gel. The spacer gel consisted of 2.03 mL of acrylamide stock A (48% acrylamide, 1.5% bis-acrylamide), 3,33 mL gel buffer, and water to a final volume of 10 mL. To polymerize the spacer gel, 33.3 μL of 10% APS and 3.33 μL of TEMED were added to the gel mixture. The spacer gel was overlayed with water, which was removed once the gel had set. The stacking gel was prepared using 0.5 mL of acrylamide stock A, 1.55 mL of gel buffer, and water to a final volume of 6.25 mL. The volumes given above were sufficient for one gel poured using 8.3 x 10.2 cm glass plates (BIO-RAD). Protein extracts electrophored in the gel were prepared in 1X sample buffer (4% SDS, 12% glycerol, 50 mM Tris-HCl pH 6.8, 2% β-mercaptoethanol, 0.01% Serva blue G). Separate anode (10X stock: 2M Tris-HCl pH 8.9) and cathode (10X stock: 1M Tris, 1M Tricine, 1% SDS; pH should be 8.25) buffers were used during electrophoresis. Gels were electrophoresed for three and a half hours at 100 V. Gel transfer, antibody probing, and detection were as described in the Western blotting section.

STRAINS AND MEDIA: YEAST AND BACTERIAL

The yeast strains used in experimental procedures include the wild-type yeast strains, W303 (α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1*) and aW303 (a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1*), and the *cox17* null mutant strains, W303ΔCOX17 (α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Δcox17::TRP1*) and aW303ΔCOX17 (a *ade2-*

1 his3-1,15 leu2-3,112 trp1-1 ura3-1 Δcox17::TRP1). Rho^0 tester strains used include KL14ρ^o (α *met6*) and CB11ρ^o (a *ade1*).

YPD (1% yeast extract, 2% peptone, 2% glucose, 2% agar), a rich glucose medium, was used as a general solid growth medium for the various yeast strains. Prior to yeast transformation, cultures were grown in liquid YPD medium (1% yeast extract, 1% peptone, 2% glucose). After yeast transformation, cells were plated on WO medium (0.67% nitrogen base without amino acids, 2% glucose, 2% agar). Appropriate amino acids (combinations of histidine, adenine, leucine, uracil, and tryptophan) were spread on WO plates to select for transformants after yeast transformation. To test for growth on nonfermentable carbon sources, yeast were plated on EG (1% yeast extract, 2% peptone, 2% glycerol, 2% ethanol, 2% agar), a rich glycerol medium. Prior to the isolation of mitochondria, yeast were grown in liquid GAL medium (1% yeast extract, 1% peptone, 2% galactose) to induce mitochondrial proliferation.

E. coli strains in liquid culture were grown in LB medium (0.5% yeast extract, 1% tryptone, 0.5% NaCl, 0.1% glucose). When strains were being grown for plasmid isolation using the Qiagen Plasmid Midi Kit, the amount of NaCl in the liquid LB medium was increased to 1%. Strains cultivated on solid medium were grown on LB plates (0.5% yeast extract, 1% tryptone, 0.5% NaCl, 0.1% glucose, 2% agar) containing either ampicillin (50 μg/mL; Sigma) or chloramphenicol (20 μg/mL; Sigma).

CELL BIOLOGY TECHNIQUES

COS-1 Cell Transfection and Immunofluorescence

COS-1 cells were plated on glass coverslips at a density of 2 x 10^5 cells per 35 mm tissue culture plate, 24 hours prior to transfection. Plasmid DNA used for

transfection was isolated using the Qiagen Plasmid Midi Kit according to the manufacturer's instructions. At the end of the plasmid isolation, the DNA was eluted in 500 µL of water and concentrated to a final volume of 100 µL in the SpeedVac Plus (Savant). Two hundred µL of Dulbecco's Modified Eagle Medium (DMEM; Sigma) was added to a sterile 1.5 mL microcentrifuge tube. Three µL of FuGENE 6 transfection reagent (Roche) was added directly into the DMEM. After mixing gently by tapping the tube, the contents of the tube were allowed to incubate at room temperature for five minutes. The DMEM/FuGENE 6 mixture was added to a sterile 1.5 mL microcentrifuge tube containing 900 ng of pCDNA3.1-hCOX17NFL. After mixing gently, the solution was left to incubate for 30 minutes at room temperature, then added dropwise to the tissue culture plate, which was swirled gently to ensure even dispersal of the transfection mixture. The plate was incubated at 37°C for 48 hours before immunofluorescence experiments were carried out.

A 1 mM stock solution of MitoTracker Red (Molecular Probes) was prepared by dissolving the contents of one tube in 94 µL dimethyl sulfoxide (DMSO; Caledon). The stock solution was diluted to 30 nM in Dulbecco's Modified Eagle Medium (DMEM; Sigma) containing fetal bovine serum. After removing the DMEM from the transfected and untransfected COS-1 cells in the tissue culture hood, one mL of 30 nM MitoTracker Red was added to each 35 mM plate and the plates were incubated at 37°C for 20 minutes in the tissue culture incubator. To fix the cells, the MitoTracker Red solution was removed and replaced with one mL of 4% paraformaldehyde (Sigma; made fresh) Following a 15 minute incubation at room temperature, the cells were washed twice with phosphate-buffered saline (PBS; pH 7.4). The cells were permeabilized for two minutes

at room temperature with cold 0.1% Triton X-100 (VWR) in PBS (made fresh, kept on ice), followed by four washes in PBS. Before antibody staining, the cells were blocked for 1 hour at room temperature by adding 80 µL of 4% normal donkey serum (Jackson ImmunoResearch) to each coverslip. After removing the blocking serum, 80 µL of primary antibody diluted in 4% donkey serum in PBS was added to each coverslip. Anti-FLAG antibody (Sigma) was used at a dilution of 1:350 (starting concentration of 4.9 µg/mL). The anti-cytochrome *c* antibody (BD PharMingen) was diluted to 1:150 for use. The cells were left to incubate with the primary antibody for one hour at room temperature in the dark. Following incubation with primary antibody, the coverslips were washed four times with PBS. Eighty µL of donkey anti-mouse FITC-conjugated secondary antibody (Jackson ImmunoResearch) that was diluted 1:200 in 4% donkey serum in PBS was added to each coverslip. After one hour at room temperature in the dark, the cells were washed four times in PBS. Coverslips were mounted face-down on glass microscope slides using 25 µL ProLong Antifade reagent (Molecular Probes; prepared according to manufacturer's instructions). The glass slides were covered with aluminum foil and allowed to dry in the dark at room temperature overnight. Once dry, the slides were kept in the dark at 4°C.

Immunofluorescence was visualized using a LSM510 laser scanning confocal microscope mounted on a Zeiss Axiovert 100M microscope. The FITC staining was visualized by excitation at 488 nm, with the fluorescence signal collected using a 505-530 nm band pass filter. The MitoTracker Red signal was induced by excitation at 543 nm, with the fluorescence signal collected using a 560 nm long pass filter.

COS-1 Cell Transfection and Subcellular Fractionation

COS-1 cells were plated at a density of 2.25 x 10^6 cells per 150 mm tissue culture plate, 24 hours prior to transfection. Plasmid DNA used for transfection was prepared as described above in the section outlining COS-1 cell transfection and immunofluorescence. In the tissue culture hood, 2.33 mL of Dulbecco's Modified Eagle Medium (DMEM; Sigma) were added to a sterile tube. Sixty μL of FuGENE 6 transfection reagent (Roche) were added directly into the DMEM. After mixing gently by tapping the tube, the contents of the tube was allowed to incubate at room temperature for five minutes. The DMEM/FuGENE 6 mixture was added to a sterile tube containing 7.5 μg of pCDNA3.1-hCOX17NFL. After mixing gently, the solution was left to incubate for 30 minutes at room temperature. The solution was added dropwise to the tissue culture plate, which was swirled gently to ensure even dispersal of the transfection mixture. The tissue culture plate was incubated for 48 hours at 37°C before subcellular fractionation experiments were carried out.

After removing the DMEM from three 150 mm plates of confluent COS-1 cells, the plates were rinsed with 5 mL sucrose buffer (0.25 M sucrose, 20 mM Tris, imM EDTA; pH at 7.4; store at 4°C). Two mL of sucrose buffer (at 4°C) were added to the first plate, which was scraped using a disposable cell lifter (Costar). The sucrose buffer, which was used to scrape the second and third plates sequentially, was transferred to the bottom of a chilled Teflon-on-glass homogenizer. The plates were scraped a second time with an additional two mL of sucrose buffer, which was also added to the homogenizer after the scraping of the third plate. The collected cells were homogenized (30 strokes) on ice and transferred to a 17 X 100 mm polypropylene sterile tube (Simport). The homogenizer was rinsed with one to two mL of sucrose buffer to collect any remaining

cells. The cells were centrifuged at 3000 rpm for seven minutes at 4°C in a SS-34 rotor in a Sorvall RC 5B Plus centrifuge. The supernatant was transferred to a fresh tube and centrifuged again at 3000 rpm for seven minutes at 4°C. After transferring to a new tube, the supernatant was centrifuged at 10 000 rpm for 10 minutes at 4°C. The post-mitochondrial supernatant, which represents the cytosolic fraction, was carefully decanted into a fresh tube and kept on ice. Seventy-five µL of the protease inhibitor, phenylmethylsulfonyl fluoride (PMSF; 10 mg/mL; Sigma), were added to the post-mitochondrial supernatant. The pellet, which represents the mitochondrial fraction, was resuspended in two mL of sucrose buffer before the volume of the tube was brought up to 10 mL. The tube was centrifuged at 10 000 rpm for 10 minutes at 4°C. After removing the supernatant, the pellet was resuspended in 0.5 mL of sucrose buffer and transferred to a 1.5 mL microcentrifuge tube. The centrifuge tube was rinsed with an additional 0.5 mL of sucrose buffer, which was added to the microcentrifuge tube. The tube was centrifuged at 13 000 rpm for 10 minutes in a microcentrifuge at 4°C. After removing the supernatant, the mitochondrial pellet was resuspended in 120 µL of sucrose buffer. Five µL of PMSF (10 mg/mL) were added to the mitochondrial suspension. Before determining the concentration of the protein in the two fractions, one mL of the post-mitochondrial supernatant fraction was concentrated to 100 µL by placing the sample in the SpeedVac Plus (Savant) for approximately 2 hours with no heat.

Yeast Whole Cell Lysate

Ten mL of liquid YPD medium in a 50 mL conical tube were inoculated with a yeast strain and incubated overnight at 30°C with shaking (225 rpm). The cells were harvested at 2500 rpm for seven minutes in a Beckman J6-MI centrifuge. After pouring

off the supernatant, the cells were washed with 1.2 M sorbitol for seven minutes at 2500 rpm. The supernatant was removed and the cells were resuspended in 600 µL of digestion buffer (1.2 M sorbitol, 75 mM NaP_i pH 7.5, 1 mM EDTA, 1% β-mercaptoethanol, and 0.45 mg/mL Zymolyase [Seikagaku]). After a one-and-a-half hour incubation at 37°C, 600 µL of 1.2 M sorbitol was added and the tube was centrifuged at 6000 rpm for 10 minutes. The cells were washed two more times with 1.2 M sorbitol. The supernatant was poured off and 20 mM Tris, pH 7.5, was added to a volume of 1 mL. The cell suspension was transferred to a 1.5 mL microcentrifuge tube and sonicated for 20 seconds on ice using the Sonic Dismembrator 60 (Fisher Scientific). After sonication, 1 µM PMSF (Sigma), 1 µg/mL leupeptin (Roche), and 1 µg/mL pepstatin (Boehringer Mannheim) were added. The tube was centrifuged at 2500 rpm for five minutes at 4°C in a microcentrifuge (Eppendorf) to sediment cellular debris. The supernatant was transferred to a fresh 1.5 mL microcentrifuge tube.

Preparation of Intact Yeast Mitochondria by Zymolyase

Ten mL of liquid GAL medium were inoculated with a yeast strain and incubated overnight at 30°C with shaking (225 rpm). Two mL of the overnight culture were used to inoculate 50 mL of GAL, which was incubated overnight at 30°C with shaking. After inoculating 800 mL of GAL with the 50 mL culture, the flask was incubated overnight at 30°C with shaking. The 850 mL culture was poured into a one L centrifuge bottle and harvested at 4200 rpm in a Beckman J6-MI centrifuge for 10 minutes. After pouring off the medium, the yeast cells were resuspended in 150 mL of 1.2 M sorbitol and transferred to a pre-weighed 250 mL centrifuge bottle. The one L centrifuge bottle was rinsed with an additional 50 mL of 1.2 M sorbitol, which was added to the 250 mL centrifuge bottle.

The centrifuge bottle containing the resuspended yeast cells was centrifuged at 6000 rpm for 5 minutes at 4°C in a SLA-1500 rotor (Sorvall). After pouring off the supernatant, the cells were resuspended in digestion buffer (1.2 M sorbitol, 0.075 M NaP_i pH 7.5, 0.1% β-mercaptoethanol, 0.5-0.7 mg/mL Zymolyase [Seikagaku]) at a concentration of 3 mL of buffer per gram of cells (wet weight). Cells were incubated at 37°C until most of the cells had been converted to spheroplasts (typically, 60-90 minutes for respiration-deficient strains and two-three hours for wild-type strains). After digestion, 1.2 M sorbitol was added to the centrifuge bottle to a final volume of 200 mL. The cells were centrifuged at 6000 rpm for 10 minutes at 4°C. Using sufficient sorbitol buffer (approximately 150 mL) to remove the Zymolyase, the cells were washed twice more with 1.2 M sorbitol. Following the last wash, the spheroplasts were resuspended in STE buffer (0.5 M sorbitol, 20 mM Tris pH 7.5, 0.5 mM EDTA) at a concentration of three to five mL of buffer per gram of starting cells. The cells were homogenized for 20 seconds in a Waring blendor. One hundred μL of PMSF (10 mg/mL) were added to the homogenized cells, which were poured into 50 mL centrifuge tubes. Using the SS-34 rotor (Sorvall), cells were centrifuged at 2500 rpm for 10 minutes at 4°C. The supernatant was collected and centrifuged again at 2500 rpm for 10 minutes in a fresh tube. The cleared supernatant was transferred to a new tube and centrifuged at 12 000 to 15 000 rpm for 15 minutes (4°C) to sediment the mitochondria. The post-mitochondrial supernatant was collected and kept on ice. The mitochondrial pellet was washed three times in STE buffer by centrifuging at 12 000 rpm for 10 to 15 minutes. After the final wash, the mitochondria were resuspended in 200 to 300 μL of 20 mM Tris, pH 7.5. Ten μL of PMSF (10 mg/mL) were added to the resuspended mitochondria. Before

determining the protein concentration of the mitochondrial and post-mitochondrial supernatant fractions, one mL of the post-mitochondrial supernatant fraction was centrifuged in a polyallomer thick wall ultracentrifuge tube (13 X 56 mm; Beckman) at 40 000 rpm for 20 minutes at 4°C in to sediment any broken mitochondria. The post-mitochondrial supernatant was removed to a fresh tube.

BIOCHEMICAL TECHNIQUES

Cytochrome Oxidase Activity Assay: Yeast Mitochondria

A cytochrome c solution was prepared by dissolving horse heart cytochrome c (Sigma) in 20 mM Tris, pH 7.5, at a concentration of 10 mg/mL. A portion of the cytochrome *c* solution was reduced with sodium hydrosulfite (Sigma). To one cuvette (QS 1000; Hellma), 0.92 mL of 20 mM KP_i, pH 7.0, and 80 µL of cytochrome c were added. This cuvette was placed in the rear cell holder of a Shimadzu UV-Visible UV-1601 PC spectrophotometer. Mitochondria to be assayed were diluted to a concentration of 10 µg/µL in 20 mM Tris, pH 7.5, then to 5 µg/µL in 0.5% deoxycholate. Using a plumper, five µL of the diluted mitochondria (i.e. 25 µg total) were added to a cuvette in the front cell holder of the spectrophotometer, which contained 0.92 mL of 20 mM KP_i, pH 7.0, and 80 µL of reduced cytochrome c. After mixing, the COX activity of the sample was determined using the Kinetics mode of the Shimadzu UVPC software. Mitochondria were diluted in the 0.5% deoxycholate right before they were assayed. All strains studied were assayed in duplicate on at least two different occasions and assayed at the same time as a wild-type yeast strain and a *cox17* null mutant strain.

Yeast Mitochondrial Cytochrome Spectra

To extract the mitochondrial cytochromes, 100 mg of KCl were dissolved in water and mixed with 100 µL 1 M Tris, pH 8.0, 13 mg of mitochondria, and 200 µL 10% deoxycholate in a final volume of 2 mL. The sample was centrifuged in a polyallomer thick wall ultracentrifuge tube (13 X 56 mm; Beckman) at 40 000 rpm for 20 minutes at 4°C. The supernatant was transferred to a 13 X 100 mm borosilicate tube (Fisher), to which 100 µL of 20% cholate was added. The contents of the tube were mixed by inversion and split between two cuvettes, which were placed in the rear and front cell holders of a Shimadzu UV-Visible UV-1601 PC spectrophotometer. The sample in the rear cuvette was oxidized with 100 µL of potassium ferricyanide (Sigma), while the sample in the front cuvette was reduced with sodium hydrosulfite (Sigma). The oxidized minus reduced spectra of the extracted cytochromes were recorded using the Spectra mode of the UVProbe Shimadzu software program. All spectra from yeast strains studied were recorded at the same time as spectra from a wild-type yeast strain and a *cox17* null mutant strain.

Protein Determination by the Folin Procedure

Protein concentration was determined according to the method described by Lowry *et al.* (Lowry et al., 1951).

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Chapter 3. Characterization and localization of human *COX17*

Material from this chapter has been previously published.

Punter, F.A., Adams, D.L., and Glerum, D.M. (2000) Characterization and localization of human *COX17*, a gene involved in mitochondrial copper transport. Hum Genet 107: 69-74.

INTRODUCTION

Cytochrome oxidase (COX) is the terminal electron acceptor of the mitochondrial respiratory chain and contributes to the proton motive force that drives the synthesis of ATP (Capaldi, 1990). This multi-subunit enzyme is remarkably conserved between lower and higher eukaryotes and consists of 13 polypeptide subunits (Saraste, 1990; Taanman and Capaldi, 1992). The three largest subunits are encoded in mitochondrial DNA and form the catalytic core of the enzyme. They contain the three copper atoms and two heme A molecules that serve as prosthetic groups in the holoenzyme and are directly involved in electron transfer. The remaining ten subunits of the COX holoenzyme are presumed to play a structural role and are encoded by nuclear DNA. In addition to the constituent subunits, there are a host of nuclear gene products that are required for assembly of the holoenzyme (McEwen et al., 1986; Tzagoloff et al., 1990). These assembly factors encompass proteins involved in expression of the individual subunits and in the provision of the requisite prosthetic groups to the apoenzyme. Studies in yeast have shown that these assembly proteins are essential components of the cytochrome oxidase biosynthetic pathway (Glerum et al., 1997). One such assembly factor is Cox17p (Cox17 protein, according to yeast nomenclature), which is a small polypetide presumed to shuttle copper from the cytosol into the mitochondrial intermembrane space (Glerum et al., 1996a). *COX17* was first identified and characterized in a yeast COX deficient mutant in which the respiration deficient phenotype is characterized by an inability to complete cytochrome oxidase assembly. It appears that the failure to assemble a functional enzyme in *cox17* mutants is probably due to an inability to provide the apoenzyme with its required copper atoms (Beers et al., 1997; Srinivasan et al., 1998).

Specific deficiencies of cytochrome oxidase in humans have been described over the last two decades (DiMauro et al., 1987; Robinson et al., 1987), with most cases being associated with severe neonatal or infantile lactic acidosis and early death. Biochemical studies of COX deficient patients suggested that a failure to assemble the holoenzyme might underlie the majority of inherited COX deficiencies (Glerum et al., 1988; Lombes et al., 1991; Adams et al., 1997) and mutations in five assembly factor genes have now been identified. Mutations in the *SURF1* gene have been shown to underlie the COX deficiency in patients with Leigh syndrome (Tiranti et al., 1998; Zhu et al., 1998). Patients with a fatal cardioencephalomyopathy or hypertrophic cardiomyopathy, marked by a severe COX deficiency, have been shown to harbor mutations in the *SCO2* gene (Papadopoulou et al., 1999; Jaksch et al., 2000). Mutations in *SCO1* have been demonstrated to cause neonatal encephalopathy and hepatic failure, in conjunction with severe COX deficiency (Valnot et al., 2000a). Lesions in *COX10* have been found in patients with isolated COX deficiency, leukodystrophy, and tubulopathy (Valnot et al., 2000b). Most recently, mutations in *COX15* have also been shown to underlie an autosomal recessively inherited COX deficiency associated with fatal infantile hypertrophic cardiomyopathy (Antonicka et al., 2003). In all instances, previous work with the corresponding yeast homologues had demonstrated a role for these proteins in respiratory chain complex assembly (Nobrega et al., 1990; Glerum et al., 1996b; Glerum et al., 1997; Mashkevich et al., 1997).

Because *COX17* may also be involved in human COX deficiency, I have characterized and localized *COX17* in the human genome. The human *COX17* orthologue was originally identified by functional complementation of a yeast *cox17* null

mutant (Amaravadi et al., 1997), but until recently, was not mapped in the human genome. In this chapter, the genomic structure, expression analysis, and chromosomal localization of the *COX17* gene and a related processed pseudogene, *COX17P*, are presented.

RESULTS

Southern Blot Analysis of *COX17*

To determine the genomic structure of *COX17*, a number of clones harboring the gene were obtained. A single P1 and six BAC clones were obtained by screening genomic libraries with the *COX17* cDNA. DNA from each of the clones was purified and two to five μg were digested with *Eco*RI, *Hind*III and both enzymes together. Subsequent Southern blot analysis using the *COX17* cDNA as a probe suggested the presence of two copies of *COX17* in the human genome. As seen in Figure 3-1A, digestion of genomic DNA with *Eco*R1 and *Hind*III together yields two fragments. The smaller fragment, which is approximately 1.2 kb in size, is also present in the double digest lane of BAC 274K10 (Figure 3-1B). The larger fragment in the double digest lane of the genomic DNA Southern blot, which is approximately 2.3 kb in size, is present in digested BAC 271E16 DNA (Figure 3-1C). These two distinct banding patterns were corroborated in the other BAC clones examined.

Genomic Structure of *COX17* and *COX17P*

Results from the Southern analysis suggested that several of the BAC clones contained similar or identical inserts and one representative clone for each of the two banding patterns was selected for further study. DNA sequencing subsequently

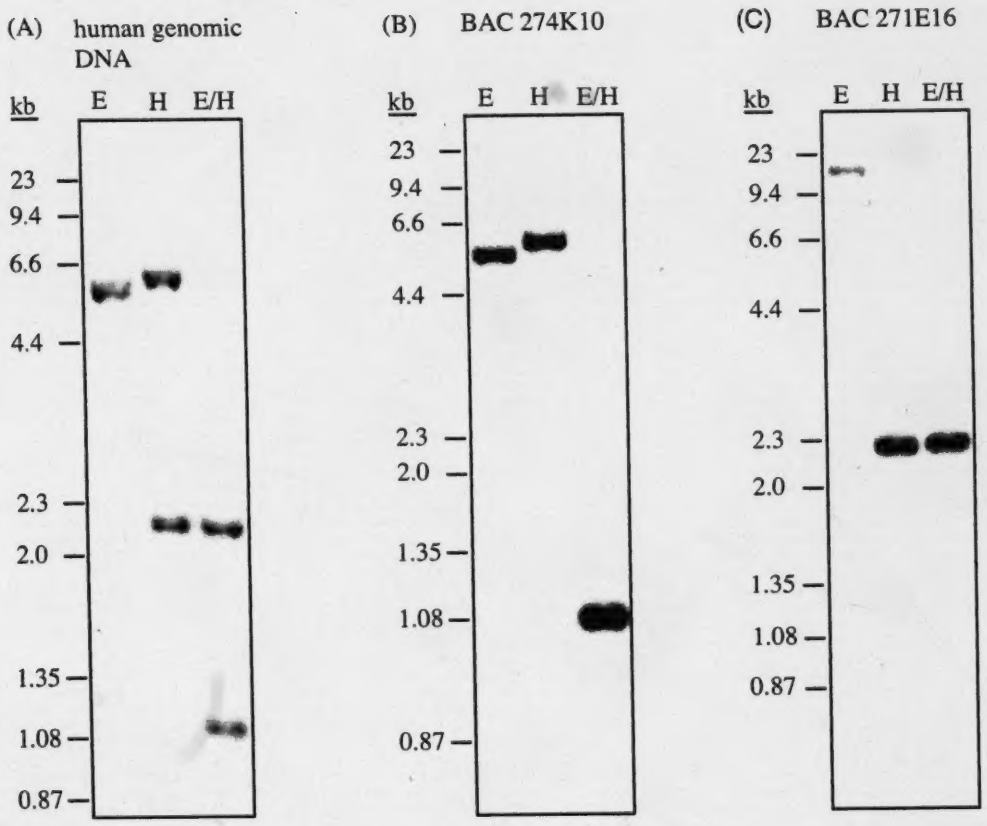

Figure 3-1. Southern blot analysis of *COX17*. The *COX17* cDNA was hybridized to human genomic DNA (A), BAC 274K10 DNA (B), and BAC 271E16 DNA (C). The migration of DNA markers is indicated on the left side of each blot. E = DNA digested with *Eco*R1; H = DNA digested with *Hin*dIII; E/H = DNA digested with *Eco*RI and *Hin*dIII.

confirmed that there are two distinct copies of *COX17* in the human genome, which we have denoted *COX17* and *COX17P*.

COX17 contains coding sequence that is identical to the published cDNA sequence, in addition to previously undescribed intronic sequence. The *COX17* gene consists of three exons and two introns and is depicted in schematic form in Figure 3-2A. Partial sequencing of the intronic DNA verified that the intron-exon boundaries conform to the canonical GT/AG rule. The approximate size of the first intron was determined by PCR, using primers that anneal to the 3' end of exon 1 and the 5' end of exon 2. A PCR product of approximately 2 kb was generated (data not shown). Interestingly, the 5' end of the first intron revealed sequence that is rich in a CTGCCCT repeat (data not shown), which is not recognized by RepeatMasker (from http://www.dot.imgen.bcm.tmc.edu:9331/). The significance of this motif is currently unknown. PCR with primers that anneal to the 3' end of exon 2 and the 3' end of intron 2 (primer 18110-13; 5'-gccacttcctgtggaaag-3') demonstrated that intron 2 spans approximately 5.5 kb of DNA (data not shown). Taken together, these data indicate that the *COX17* gene is contained in approximately 8 kb of genomic DNA.

COX17P does not contain any intronic DNA and several nucleotide changes exist between the gene and the cDNA. Within the 5' UTR, the first 13 nucleotides of *COX17P* and the *COX17* cDNA do not share any identity. A nucleotide difference also exists at position -64, where a C→T transition is present in *COX17P* (nucleotides are numbered as in the published cDNA sequence; (Amaravadi et al., 1997)). In addition to the differences found in the 5' UTR, four nucleotide changes, consisting of 32 C→T (Pro→Leu), 53 A→G (Lys→Arg), 73 G→A (Ala→Thr) and 96 G→A (silent), are found

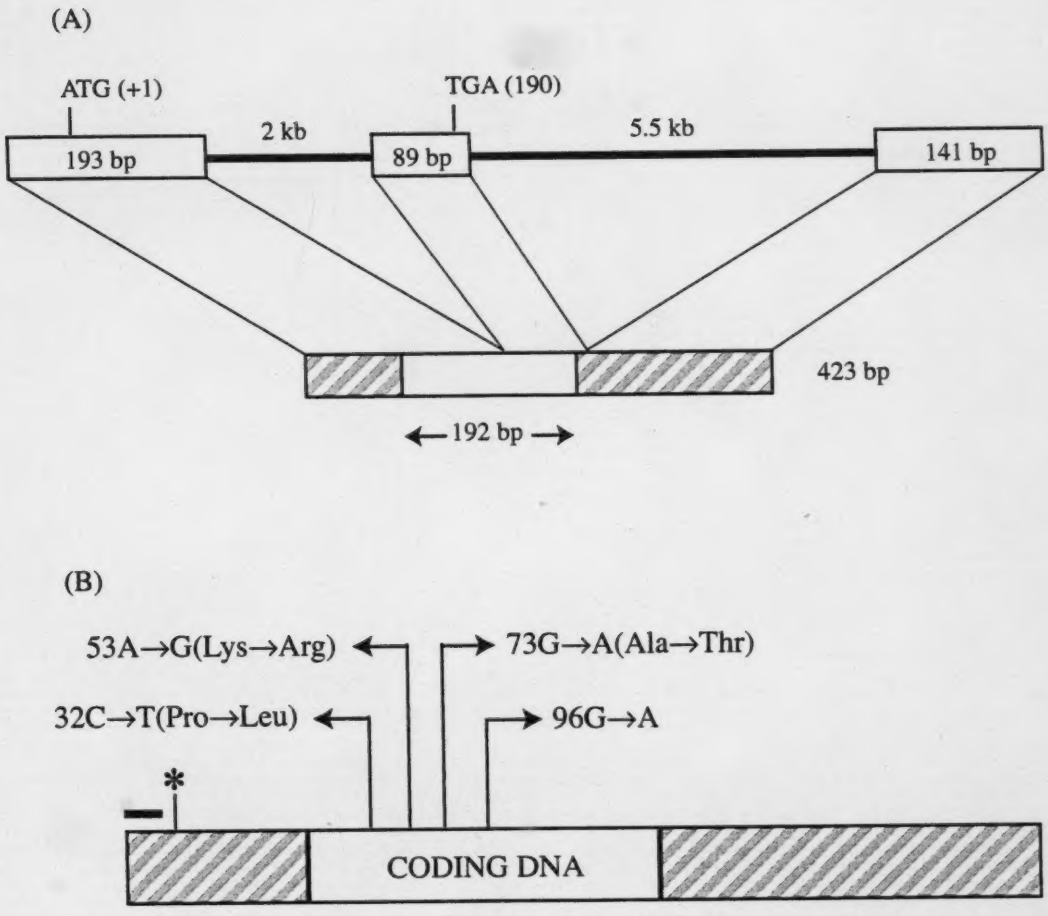

Figure 3-2. Organization of *COX17* and *COX17P*. (A) A schematic representation of the *COX17* gene and the corresponding cDNA is shown. In the *COX17* gene, exons are depicted as white boxes (exon size is shown within the box), while introns are represented as solid black lines. The first methionine codon at nucleotide position +1 is shown, as is the stop codon at nucleotide position 190. Within the *COX17* transcript, the hatched lines represent the untranslated region, while the white box represents the coding region. The length of the *COX17* transcript does not include the polyA tail. The lines joining the gene and cDNA indicate how the three exons contribute to the transcript. The diagram is not to scale. (B) *COX17P* is similar, but not identical to the *COX17*. The four nucleotide differences occurring within the coding region are shown. Hatched lines represent non-coding DNA. The bar above the gene represents the nucleotide differences at the 5' end of the *COX17P*. The asterisk indicates the nucleotide difference at position 23.

within the coding region (Figure 3-2B). As shown in Figure 3-3, the nucleotide difference at position 73 occurs within the putative copper-binding domain of COX17 at an alanine residue that is conserved in all mammalian COX17 proteins identified to date. None of the *COX17* ESTs in the database (GenBank; http://www.ncbi.nlm.nih.gov/) contain the nucleotide changes seen in *COX17P*, which also has the polyadenylation signal seen in the cDNA. These results suggest that *COX17P* is a processed pseudogene.

Sequence information was submitted to GenBank (GenBank; http://www.ncbi.nlm.nih.gov/) and can be accessed using the accession numbers AF269243, AF269244, and AF269245.

Expression of *COX17*

A single transcript was detected when the *COX17* cDNA was hybridized to a human multiple tissue Northern blot. As seen in Figure 3-4, *COX17* appears to be most highly expressed in tissues with a high aerobic demand. Although not clear from the figure, longer exposure revealed the presence of *COX17* mRNA in all lanes, suggesting that it is ubiquitously expressed, as expected for a gene that is involved in an essential process such as oxidative phosphorylation. Because the *COX17* transcript was smaller than the lowest marker on the commercial Northern blot, the blot was also probed with the *S26* cDNA (Vincent et al., 1993). The *COX17* cDNA hybridizes to a transcript that migrates below the 700 bp *S26* transcript, consistent with an expected transcript size of approximately 450 bp.

To determine whether both *COX17* and *COX17P* are expressed, RT-PCR was performed with RNA isolated from a lymphoblastoid cell line. Using primers COX17H-1 (specific to the 5' end of *COX17* and the *COX17* cDNA) and COX17H-4, a

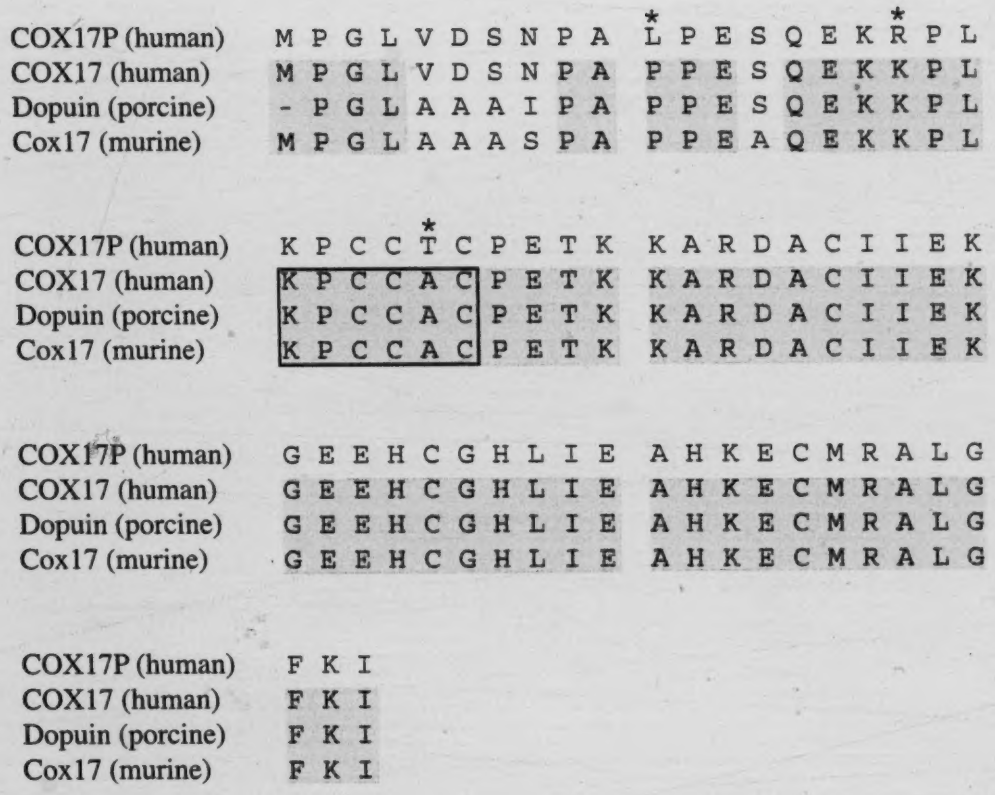
COX17P (human) M P G L V D S N P A L P E S Q E K R P L
COX17 (human) M P G L V D S N P A P P E S Q E K K P L
Dopuin (porcine) - P G L A A A I P A P P E S Q E K K P L
Cox17 (murine) M P G L A A A S P A P P E A Q E K K P L
COX17P (human) K P C C T C P E T K K A R D A C I I E K
COX17 (human) K P C C A C P E T K K A R D A C I I E K
Dopuin (porcine) K P C C A C P E T K K A R D A C I I E K
Cox17 (murine) K P C C A C P E T K K A R D A C I I E K
COX17P (human) G E E H C G H L I E A H K E C M R A L G
COX17 (human) G E E H C G H L I E A H K E C M R A L G
Dopuin (porcine) G E E H C G H L I E A H K E C M R A L G
Cox17 (murine) G E E H C G H L I E A H K E C M R A L G
COX17P (human) F K I
COX17 (human) F K I
Dopuin (porcine) F K I
Cox17 (murine) F K I

Figure 3-3. Alignment of the predicted amino acid sequences of homologous mammalian COX17 proteins ("dopuin" is the original name given to porcine Cox17). Identical residues in the three expressed proteins are highlighted by grey shading. Asterisks denote amino acid differences in human COX17P. The black box indicates the putative copper-binding domain. Sequence references are noted in the text.

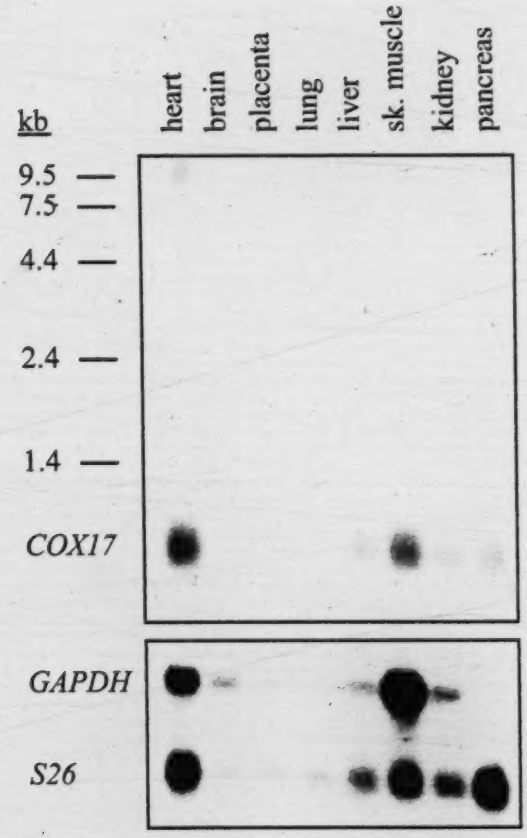

Figure 3-4. Northern blot analysis of *COX17*. The *COX17* cDNA was hybridized to a human multiple tissue Northern blot (Clontech). The blot was also probed with the *GAPDH* cDNA (1.3 kb) and the *S26* cDNA (0.7 kb). The migration of RNA markers is indicated on the left side of the blot. Sk. muscle = skeletal muscle.

350 bp product was amplified from the lymphoblastoid cDNA pool (*COX17*, + RT, Figure 3-5). When the same experiment was carried out using primers COX17B-2 (specific for the 5' end of *COX17P*) and COX17H-4, no product was amplified from the lymphoblastoid cDNA pool (*COX17P*, + RT, Figure 3-5). These primers, however, did amplify a product from BAC 271E16 and human genomic DNA (*COX17P*, Figure 3-5). These results indicate that *COX17P* is not expressed in the tissue tested and further suggest that *COX17,* and not *COX17P*, is the gene producing the single transcript seen on Northern blots. In addition to the nucleotide differences present in *COX17P*, the absence of *COX17P* expression further suggests that *COX17P* represents a processed *COX17* pseudogene.

Localization of *COX17* and *COX17P*

Radiation hybrid mapping was used to determine the chromosomal locations of both the *COX17* and *COX17P* genes. Primers COX17H-3 and COX17H-4 were used to amplify a 235 bp fragment corresponding to *COX17P*, which localizes to chromosome 13q14-q21. The *COX17* mRNA was also assigned to this chromosome 13 locus by two different consortia (RHdb RH67876 and RHdb RH 75245). However, these primers cannot amplify a 235 bp product from *COX17* since they are specific for exons 1 and 3, which are separated by approximately 6 kb of DNA. To obtain the correct chromosomal location for *COX17*, primers 18110-13 and 18110-18 were used to generate a 220 bp fragment corresponding to a portion of intron 2, which localized to the long arm of chromosome 3 (3q13.1 – q21), between the markers D3S9340 and D3S1765.

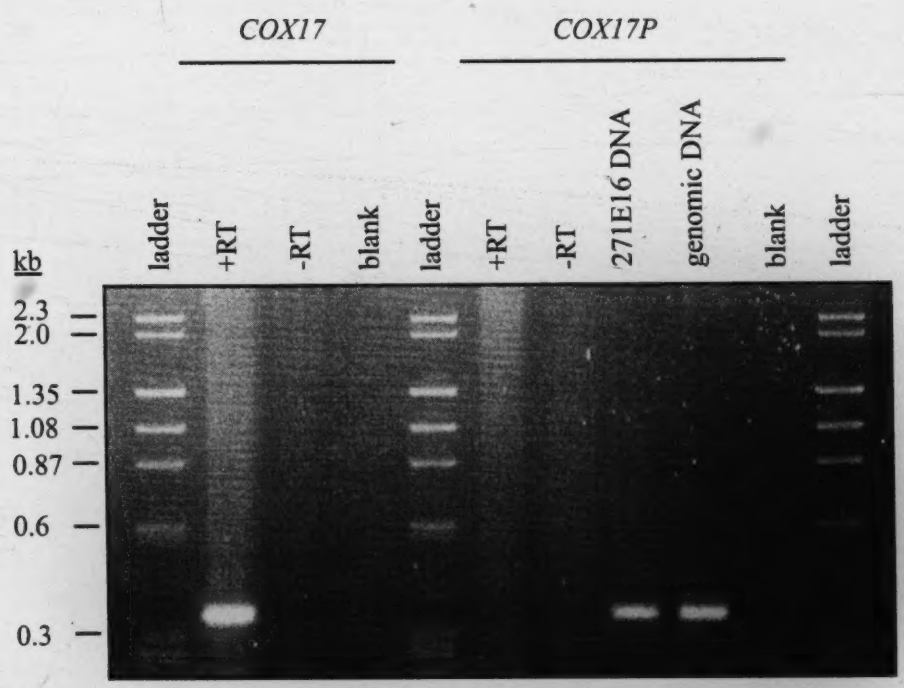

Figure 3-5. Expression studies of *COX17* and *COX17P*. RT-PCR was performed on RNA isolated from a lymphoblastoid cell line. Expression of both genes was detected by PCR using gene specific primers described in the text. Positive controls for *COX17P* included BAC 271E16 and human genomic DNA. +RT = RT-PCR carried out in the presence of reverse transcriptase; -RT = RT-PCR carried out in the absence of reverse transcriptase; blank = PCR carried out in the absence of template DNA.

DISCUSSION

COX17 is a small copper chaperone that is responsible for recruiting copper to mitochondria (Glerum et al., 1996a; Beers et al., 1997). Studies in yeast have shown that the presence of Cox17p is essential for assembly of a functional cytochrome oxidase. Our current study, which investigates the human orthologue of *COX17*, has determined that there are two distinct copies of *COX17* in the human genome. The *COX17* gene, which is ubiquitously expressed, is composed of three exons and two introns and spans approximately 8 kb of genomic DNA. *COX17P*, in contrast, does not appear to be expressed. While similar to the *COX17* cDNA, *COX17P* harbors a number of nucleotide differences, including one that would alter a conserved amino acid residue in the putative copper-binding domain of COX17. Of the over 65 human *COX17* ESTs that are available in the current database, none of the ESTs contain the same nucleotide differences as *COX17P*. Although the possiblity that *COX17P* is expressed in a tissue that hasn't been examined cannot be excluded, our results support the notion that *COX17P* is a processed pseudogene.

Two different consortia, Genethon and WTCHG, have previously localized *COX17* to chromosome 13 by radiation hybrid mapping (RHdb RH67876 and RHdb RH75245, respectively). However, the primers designed by Genethon from the *COX17* cDNA sequence would anneal at the exon 1/intron1 and intron 2/exon 3 boundaries of *COX17*. Primers at these positions would not amplify a product from the *COX17* gene. WTCHG used primers that anneal to exons 1 and 3, which could only amplify a 235 bp product from *COX17P*, given that *COX17* contains several kilobases of intervening DNA. In both cases, it appears that the processed pseudogene, *COX17P*, was mapped.

The results presented here indicate that the expressed copy of *COX17* lies on the long arm of chromosome 3, most likely in the pericentromeric region. At the time this work was carried out, the region of chromosome 3 containing *COX17* was very poorly characterized, with relatively few markers and a dearth of sequence information. More recent human genome sequencing data place *COX17* at chromosome 3q13.32 (NCBI; http://www.ncbi.nlm.nih.gov), in agreement with the location determined by radiation hybrid mapping. At present, none of the human diseases mapped to this region seem likely to be associated with a defect in COX17.

Recently, several mammalian COX17 homologs have been identified. Alignment of the predicted protein sequences of murine (GenPept accession number P56394), porcine (Chen et al., 1997) and human (Amaravadi et al., 1997) COX17 show that mouse and pig have 92% and 93% identity, respectively, with human COX17 (Figure 3-3). Along with yeast Cox17p, all three mammalian COX17 proteins share the KXCCXC motif, which is believed to bind copper and is also present in all mouse and human metallothioneins (Amaravadi et al., 1997). Currently, the precise physiological role of COX17 within mammalian cells has not been established. Functional studies must now be undertaken to delineate whether mammalian COX17 proteins act in the same fashion as their yeast counterpart. In this regard, it is interesting to note that COX17 is a member of a conserved family of cellular copper chaperones (Valentine and Gralla, 1997; Harrison et al., 1999) that all appear to traffic copper to a specific target protein within the cell. While the cellular role of COX17 is similar to that of the copper chaperones ATOX1 and CCS, it does not share any sequence similarity with these two proteins. ATOX1 chaperones copper to ATP7A and ATP7B, the Menkes (Larin et al., 1999) and

Wilson (Hamza et al., 1999) disease proteins, respectively, and may be involved in copper deficiency disorders. CCS provides copper to SOD1, the Cu,Zn superoxide dismutase, and may therefore play a role in the etiology of amyotrophic lateral sclerosis (Schmidt et al., 1999). In light of the very specialized function of these copper chaperones, further functional studies may help to determine whether COX17 is involved in the phenotypes of copper storage disorders, in addition to its potential role in inherited COX deficiencies.

Given that mutations in yeast *COX17* result in a respiration deficient phenotype, it is entirely possible that errors in mitochondrial copper recruitment also play a role in human COX deficiency. The elucidation of the genomic structure and the correct chromosomal location of the *COX17* gene provide new information on a candidate gene for inherited COX deficiencies. This information is an important supplement to the standard RT-PCR approaches currently possible, which do not allow examination of promoters or intron/exon boundaries and are prone to false positives from contamination with genomic DNA. With the information presented here, *COX17* can now be assessed to determine whether mutations in the gene are an underlying cause of some cases of human mitochondrial disease.

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Chapter 4. Conservation of COX17 function between yeast and mammals

Material from this chapter has been submitted for publication.

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INTRODUCTION

Cytochrome oxidase (COX), the terminal electron acceptor of the mitochondrial respiratory chain, is a multimeric enzyme complex located in the mitochondrial inner membrane. Mammalian COX is composed of 13 polypeptide subunits, with the three catalytic subunits (COX I-III) encoded by the mitochondrial genome and the remaining 10 subunits encoded by nuclear DNA (Saraste, 1999). In addition to the constituent subunits, a wide array of other proteins is required to facilitate the assembly of the holoenzyme. One important aspect of COX biogenesis involves the incorporation of copper into the holoenzyme, where the heavy metal plays an essential role in electron transfer. Two atoms of copper are found at the binuclear Cu_A site of COX II, which accepts electrons directly from cytochrome c. A third atom of copper is bound to COX I, constituting the Cu_B at the active site of the enzyme (Tsukihara et al., 1995).

The process of COX assembly, including prosthetic group insertion, remains poorly understood (Grivell et al., 1999). However, the study of COX-deficient mutants in the yeast, *Saccharomyces cerevisiae*, has led to the identification of three proteins involved in the provision of copper to COX. The first protein identified in this pathway was Cox17p, a small, hydrophilic protein that localizes to both the cytosol and the intermembrane space (Glerum et al., 1996; Beers et al., 1997). Because the respiration-deficient phenotype of a Δ*cox17* yeast strain could be rescued by the addition of supplemental copper to the growth medium, it was proposed that Cox17p functions as a copper chaperone, shuttling copper from the cytosol to the intermembrane space of the mitochondrion (Glerum et al., 1996). Sco1p, an inner mitochondrial membrane protein, was isolated as a multicopy suppressor of a *cox17* mutant. The genetic interaction

between *COX17* and *SCO1* implies that Sco1p acts downstream of Cox17p in the mitochondrial copper transport pathway (Glerum et al., 1996). An analysis of *sco1* mutants has suggested that Sco1p may be specific for the delivery of copper to the Cu_A site located on COX II. From studies in the bacterium, *Rhodobacter sphaeroides*, it appears that a third copper-binding protein, Cox11p, is required for copper insertion at the Cu_B site in COX subunit I (Hiser et al., 2000). Cox17p coordinates two (Beers et al., 1997; Srinivasan et al., 1998) or three (Heaton et al., 2000) atoms of copper, while Sco1p and Cox11p both bind one copper ion (Nittis et al., 2001; Carr et al., 2002).

The human *COX17* cDNA was originally isolated by functional complementation of a yeast *cox17* mutant (Amaravadi et al., 1997). There are two *COX17* loci in the human genome, one on the long arm of chromosome 3 and a processed pseudogene on chromosome 13 (Punter et al., 2000). Expression analysis of *COX17* indicates that the gene is ubiquitously expressed, although at different levels in the variety of tissues tested (Punter et al., 2000). This ubiquitous but varying pattern of expression is characteristic of all human COX assembly factors identified to date (Shoubridge, 2001).

Mammalian *COX17* homologues have also been studied in porcine small intestine (Chen et al., 1997) and a number of rodent tissues and cell lines (Nishihara et al., 1998; Kako et al., 2000). Murine *Cox17* mRNA was detected in all tissues tested, indicating that the mouse gene is also ubiquituously expressed. Immunofluorescence experiments carried out in a mouse cell line detected Cox17 in perinuclear regions, but not in mitochondria (Kako et al., 2000), suggesting that mammalian COX17 proteins do not have the same subcellular localization as their yeast counterpart. Because Cox17 appeared to localize in the vicinity of the ER and Golgi and could be detected at high

levels in cell lines from mouse brain, the authors proposed that mammalian COX17 plays a role in the synthesis or secretion of neuropeptide/peptide hormone.

Given that the human *COX17* cDNA was isolated by functional complementation of a yeast mutant, it seems likely that COX17 would have a conserved function in yeast and mammals. In this study, it is shown that human COX17 can almost fully restore COX activity and assembly when expressed in a yeast *cox17* null mutant. By two different techniques, it is also demonstrated that human COX17 is localized to the mitochondrion. Taken together, these results suggest that mammalian COX17 has a similar subcellular distribution and function as yeast Cox17p, namely in the provision of copper to the mitochondrion.

RESULTS

Human *COX17* can rescue the respiration-deficient phenotype of a yeast *cox17* null strain

Yeast strains carrying mutations in COX assembly genes are respiration-deficient and are unable to grow on nonfermentable carbon sources, such as ethanol and glycerol (Tzagoloff and Dieckmann, 1990). To determine the extent to which human *COX17* could restore growth in a yeast *cox17* null mutant, human *COX17* was cloned into a yeast expression vector, generating pMGL4-hCOX17, and transformed into the *cox17* null mutant strain. When present in multiple copies, human *COX17* was able to rescue the respiration-deficient phenotype of the Δ*cox17* strain, although the growth rate was slightly slower than that of wild-type yeast (Figure 4-1). The *cox17* null mutant strain transformed with empty pMGL4 vector did not show any growth on YEPG medium.

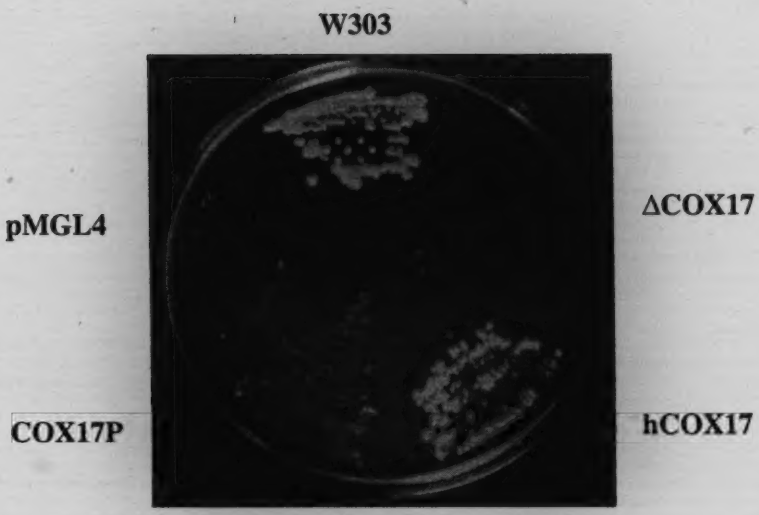

Figure 4-1. Human *COX17* can functionally complement a yeast *cox17* null mutant. Yeast transformants, along with the wild-type parent strain and the *cox17* null mutant, were streaked on YPD (rich glucose) plates and grown at 30°C for two days. Following replica plating, strains were scored for growth and photographed after 40 hours on EG (nonfermentable carbon sources) plates. W303 = wild-type parent strain; ΔCOX17 = *cox17* null mutant strain; hCOX17 = *cox17* null mutant strain transformed with a plasmid expressing human *COX17*; COX17P = *cox17* null mutant strain transformed with the human *COX17* pseudogene; pMGL4 = *cox17* null mutant strain transformed with empty plasmid.

COX17P, the human *COX17* pseudogene, encodes three amino acid changes (P11L, K18R, A25T) relative to the sequence of the human *COX17* gene. When transformed into the 'α' mating-type yeast *cox17* null mutant, the strain shows approximately one-fifth of the rate of wild-type growth on nonfermentable carbon sources. Two of the amino acid changes found in *COX17P* have been introduced into the equivalent amino acid positions in yeast Cox17p, although these alterations have no effect on respiratory growth (see Chapter 5). These results suggest that the slower growth of the pseudogene relative to the expressed gene is due to the combined effect of the mutations or due to the third amino acid substitution that has not been investigated in the yeast protein. When transformed into the 'a' mating-type yeast *cox17* null mutant strain, *COX17P* does not confer any respiratory growth. A difference in growth phenotype of several yeast *cox17* mutants was also noted in the two yeast *cox17* null mutant strains that differ only in mating type (see Chapter 5). Although the reason for the disparate phenotypes is not immediately clear, it is likely that it results from a difference in nuclear backgrounds of the two strains that has arisen over time.

Human *COX17* restores both COX activity and assembly to a *cox17* null mutant

In addition to the inability to grow on nonfermentable carbon sources, COX-deficient yeast mutants exhibit severely decreased COX activity and lack spectrally detectable cytochromes *a* and a_3. In general, the rate of respiratory growth is proportional to the residual COX activity and the amount of assembled COX holoenzyme. To ensure that the presence of human COX17 rescues the Δ*cox17* phenotype by increasing COX activity, mitochondria were isolated from the yeast strain transformed with pMGL4-hCOX17, along with the wild-type parent and *cox17* null

mutant strains. COX activity assays showed that in the strain expressing human COX17, COX activity was approximately 80% of wild-type levels (Figure 4-2), while COX activity in the Δ*cox17* strain was less than 10% of wild-type levels. Isolated mitochondria were also used for spectral analysis of the three yeast strains (Figure 4-3). Mitochondrial cytochrome spectra for the respiratory competent parent strain, W303, reveal the characteristic cytochrome aa_3 absorbance peak at 603 nm, which is absent in the *cox17* null mutant strain. In the strain expressing human COX17, there is a restoration of the 603 nm peak, indicative of assembled COX. The slightly smaller size of the peak in this strain correlates with the amount of residual COX activity.

Human COX17 localizes to the mitochondrion

There is a high degree of similarity between yeast and mammalian COX17 proteins, particularly in the spacing of the cysteine residues. In a previous study, it has been shown that three of the conserved cysteine residues are involved in the binding of copper (Heaton et al., 2000). It is clear, both from earlier work and the current results, that the human *COX17* cDNA can almost completely compensate for the lack of yeast Cox17p. Taken together, these facts strongly suggested that the subcellular distribution of yeast Cox17p (Beers et al., 1997) would be conserved in mammalian cells.

To express human COX17 in a mammalian cell line, the *COX17* cDNA sequence containing FLAG epitope DNA sequence at its 5'-end was cloned into a mammalian expression vector. The resulting construct, pCDNA3.1-hCOX17NFL, was used to transfect COS-1 cells, which were harvested and subjected to subcellular fractionation.

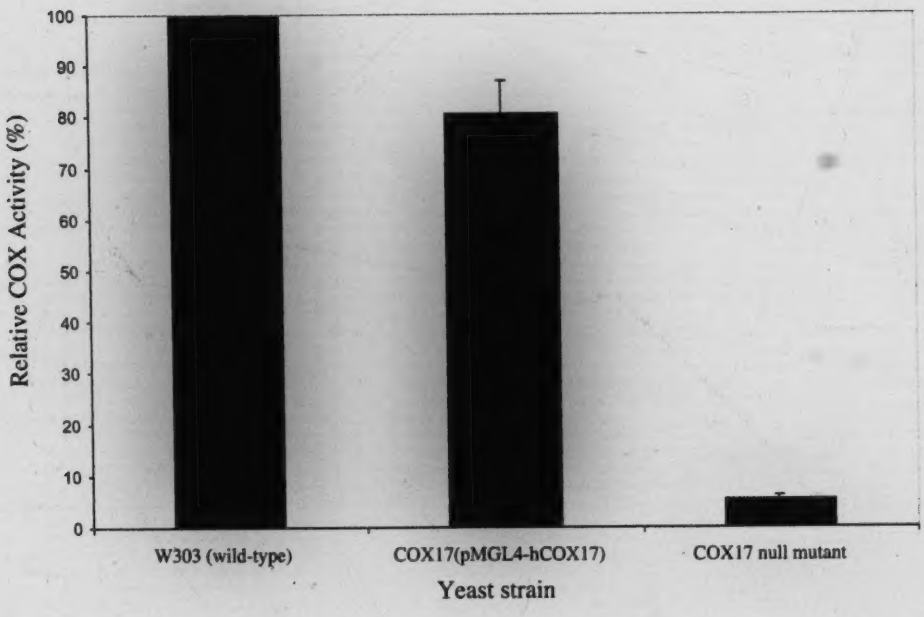

Figure 4-2. Cytochrome oxidase activity is restored by human *COX17*. Cytochrome oxidase was measured by a photometric assay with mitochondria isolated from W303, ΔCOX17, and ΔCOX17(pMGL4-hCOX17) yeast strains. Activities are expressed as a percentage of wild-type cytochrome oxidase activity and are the average of at least four assays. Lines above the bars represent standard error of the mean. W303 = wild-type yeast strain; ΔCOX17 = *cox17* null mutant yeast strain; ΔCOX17(pMGL4-hCOX17) = *cox17* null mutant yeast strain transformed with a plasmid expressing human *COX17*.

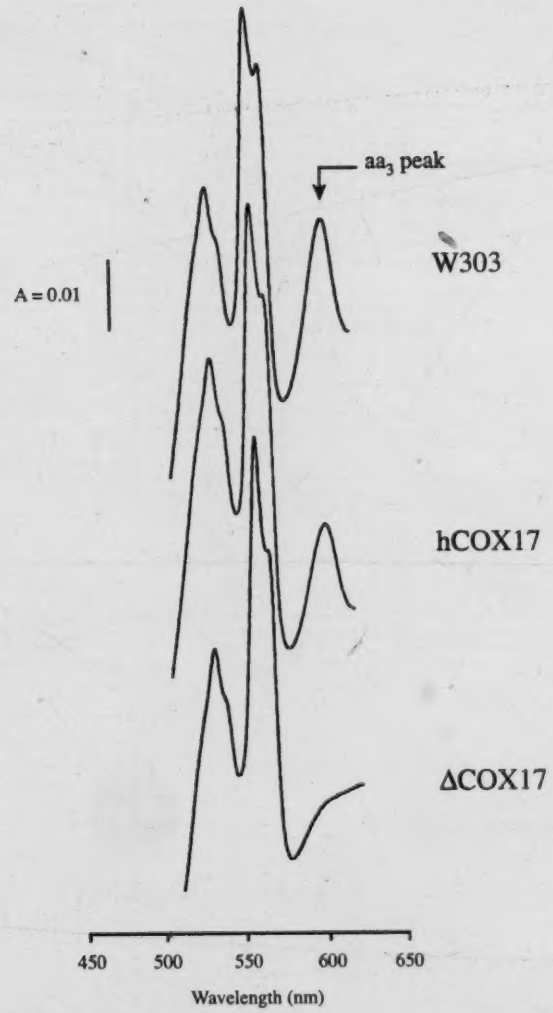

Figure 4-3. The expression of human *COX17* in a yeast *cox17* null mutant results in assembled cytochrome oxidase. Cytochrome spectra were obtained from mitochondria extracted and solubilized with 5% potassium chloride and 1% deoxycholate at a protein concentration of 6.5 mg/mL. Difference spectra of reduced *versus* oxidized extracts were obtained at room temperature. The α-band corresponding to cytochrome aa_3 has a maximum at 603 nm and is indicated by an arrow. W303 = wild-type yeast strain; ΔCOX17 = *cox17* null mutant strain; hCOX17 = *cox17* null mutant strain transformed with a plasmid expressing human *COX17*.

After separation of mitochondrial and post-mitochondrial supernatant fractions from transfected and untransfected cells on 12% polyacrylamide gels, Western blots were probed with antibody against the FLAG epitope. As shown in Figure 4-4, a novel band corresponding to the N-terminal FLAG-tagged COX17 fusion protein could be detected in both the mitochondrial and post-mitochondrial supernatant fractions of transfected, but not untransfected, COS-1 cells. The post-mitochondrial supernatant fraction contains the cytosolic cellular components. When COS-1 cells were transfected with untagged COX17, COX17 was also detected by Western blotting of these fractions with polyclonal antibodies against the N-terminus of native human COX17 (data not shown). To verify the specificity and purity of the subcellular fractionation, the two fractions were probed with antibody against exclusively mitochondrial or cytosolic marker proteins. Human COX I, the largest subunit of COX, is an integral component of the mitochondrial inner membrane, and thus serves as a distinct mitochondrial marker. Western blotting with a monoclonal antibody to COX I confirmed the presence of this protein in the mitochondrial fractions from both the transfected and untransfected cells, but not in the either of the post-mitochondrial supernatant fractions (Figure 4-4). This indicated that the presence of detectable COX17 in the cytosolic fraction is not due to a failure to pellet broken or damaged mitochondria. The level of COX1 in the transfected and untransfected cells appears identical, as the overexpression of COX17 would not be expected to lead to higher levels of the COX holoenzyme. Human farnesyltransferase, a cytosolic protein, is involved in farnesylation of a variety of intracellular protein targets (Reiss et al., 1990; Schaber et al., 1990). Immunoblotting of the same subcellular

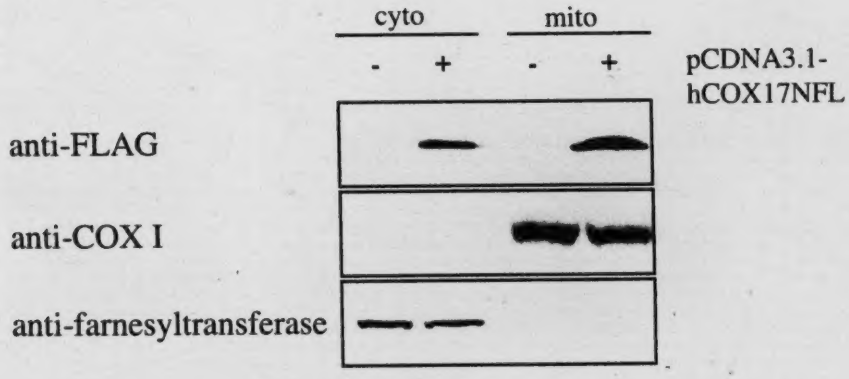

Figure 4-4. Subcellular fractionation of transfected and untransfected COS-1 cells. COS-1 cells were tranfected with pCDNA3.1-hCOX17NFL, which contains the human *COX17* gene with a FLAG epitope fused in-frame to its 5' end, and subjected to subcellular fractionation. Post-mitochondrial supernatant and mitochondrial fractions were separated on 12% polyacrylamide gels, transferred to nitrocellulose, and probed with antibodies against the FLAG epitope, human COX I (a mitochondrial protein), and human farnesyltransferase (a cytosolic protein). For the anti-FLAG blot, 30 μg of protein was loaded in each lane. On the anti-COX I and anti-farnesyltransferase blots, 40 μg of post-mitochondrial supernatant protein and 45 μg of mitochondrial protein were electrophoresed in the gels. - = untransfected COS-1 cells; + = transfected COS-1 cells; cyto = post-mitochondrial supernatant fraction; mito = mitochondrial fraction.

fractions with a monoclonal antibody (IB7) to the α subunit of the human farnesyltransferase detected a band only in the two post-mitochondrial supernatant fractions (Figure 4-4), thus demonstrating the lack of cytosolic contaminants in the mitochondrial fraction.

In order to confirm the results obtained using a biochemical fractionation approach, the localization of COX17 in transfected and untransfected COS-1 cells was also studied by immunofluorescence (Figure 4-5, Figure 4-6). Cells transfected with pCDNA3.1-hCOX17NFL, from which N-terminally FLAG-tagged COX17 is expressed, were immunostained with FLAG antibody and showed detectable FLAG-COX17 in structures surrounding the nucleus. The COS-1 cells were also incubated with the mitochondria-specific dye, MitoTracker Red CMX-Ros. When the immunofluorescence images from the FLAG antibody and MitoTracker Red were merged, the same structures detected by the FLAG antibody were stained by MitoTracker Red, indicating that FLAG-COX17 colocalizes with mitochondria. To confirm the specificity of the FLAG antibody, untransfected COS-1 cells were incubated with anti-FLAG antibody and MitoTracker Red. In this case, the MitoTracker Red staining did not overlap with the green fluorescent signal from FITC-conjugated secondary antibody (Figure 4-5). To verify the colocalization of mitochondrial protein with MitoTracker Red, untransfected COS-1 cells were also stained with anti-cytochrome c antibody and the mitochondrial specific dye. Both MitoTracker Red and antibody against cytochrome c, a mitochondrial intermembrane space protein, detected the same cellular structures (Figure 4-5).

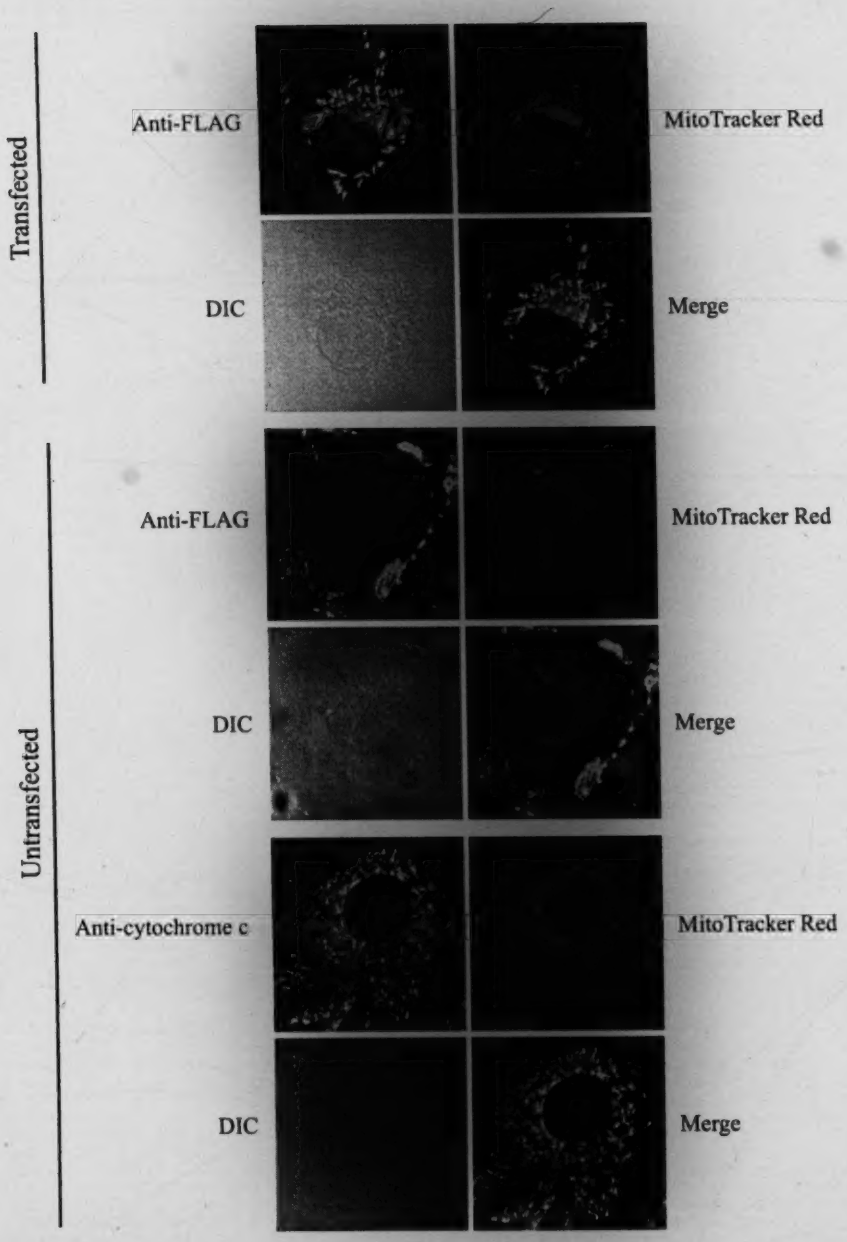

Figure 4-5. Indirect immunoflurescence of tranfected and untransfected COS-1 cells. COS-1 cells transiently transfected with pCDNA3.1-hCOX17NFL were incubated with anti-FLAG antibody (green) and MitoTracker Red (red). The yellow represents the merging of the red and green signals and indicates colocalization of epitope-tagged COX17 with mitochondria. As a negative control, untransfected COS-1 cells were incubated with anti-FLAG antibody(green) and MitoTracker Red (red). Untransfected COS-1 cells were also incubated with MitoTracker Red and antibody against cytochrome c, a known mitochondrial intermembrane space protein. DIC = differential interference contrast.

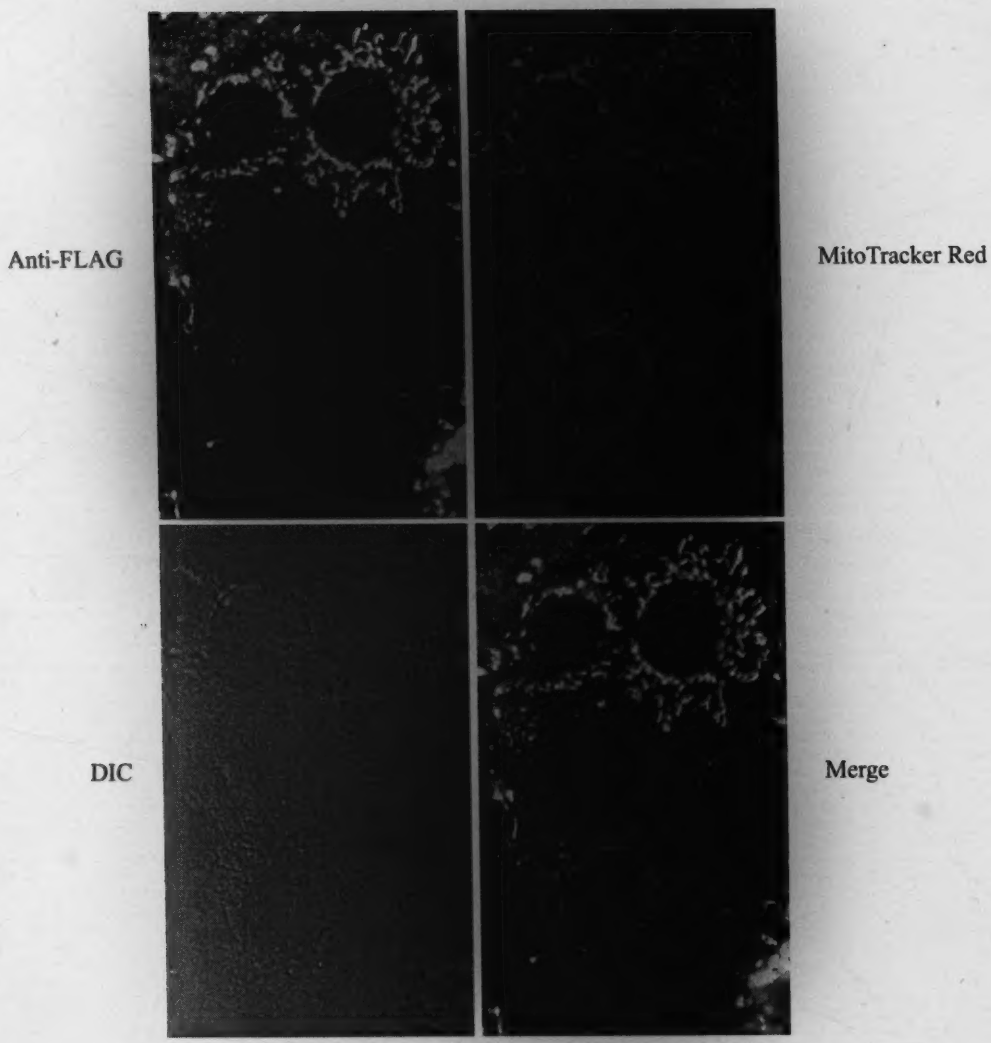

Figure 4-6. Indirect immunofluorescence of transfected COS-1 cells. COS-1 cells transiently transfected with pCDNA3.1-hCOX17NFL were incubated with anti-FLAG antibody (green) and MitoTracker Red (red). The yellow represents the merging of the red and green signals and indicates colocalization of epitope-tagged COX17 with mitochondria. DIC = differential interference contrast.

DISCUSSION

Studies in yeast have shown that Cox17p is essential for the assembly of functional COX. In spite of major advances in understanding yeast Cox17p, very little is known about the role of COX17 in mammalian cells. Because many aspects of both copper transport (Valentine and Gralla, 1997) and COX assembly appear to be highly conserved between yeast and higher eukaryotes, it seems likely that COX17 is also acting as a mitochondrial copper chaperone in the mammalian cell. When present at high copy number, human *COX17* corrects the respiration-deficient phenotype of a *cox17* null mutant strain almost as efficiently as yeast *COX17*. The presence of human *COX17* in the Δ*cox17* yeast strain restores both COX activity and assembly, demonstrating the specificity of human COX17 function. Spectral analysis of the Δ*cox17* yeast strain transformed with human *COX17* shows the presence of a detectable peak at 603 nm for cytochromes *a* and a_3. Although the area under the peak is less in the transformed strain than in the wild-type strain, the detectable peak indicates that COX assembly is occurring when human COX17 is expressed in the yeast cell. The slightly reduced levels of COX activity and assembly in yeast transformed with human *COX17*, relative to wild-type yeast, is consistent with the slightly slower growth of the transformed strain on nonfermentable carbon sources.

Because of the sequence homologies between the yeast and human COX17 proteins, as wells as the ability of human COX17 to replace yeast Cox17p, it was predicted that the subcellular distribution of COX17 in mammalian cells would parallel that seen in yeast cells. Using transient transfection of COS-1 cells with a vector expressing an epitope-tagged version of human COX17, subcellular fractionation

experiments revealed that human COX17 is present in both the mitochondrial and cytosolic fractions, similar to the distribution of Cox17p in the yeast cell. The FLAG-COX17 fusion protein was only detected in the transfected fractions, suggesting that immunostaining by the anti-FLAG antibody was specific for the epitope-tagged COX17. The purity of the mitochondrial and cytosolic fractions in the subcellular fractionation experiment was confirmed using antibodies against mitochondrial and cytosolic marker proteins. In addition, immunofluorescence experiments showed the FLAG-COX17 fusion protein was detected in cytoplasmic structures of transfected COS-1 cells. The same structures detected by the FLAG antibody were also stained by MitoTracker Red, with the overlap in fluorescent signals suggesting that structures immunostained by the FLAG antibody are mitochondria. Therefore, two independent methods demonstrate that human COX17 is a mitochondrial protein.

The mitochondrial localization of mammalian COX17 presented in this study contradicts a previous report that failed to localize murine Cox17 to the mitochondrion by immunofluorescence in AtT-20, a mouse cell line derived from corticotrophic tumour (Kako et al., 2000). The Cox17 antibody detected the protein in perinuclear regions, leading the authors to propose that Cox17 is localized in the ER and Golgi, where it could play a role in the synthesis or secretion of certain hormones. Furthermore, because the authors found that Cox17 is highly expressed in certain pituitary and neuroendocrine cell lines, they suggested that Cox17 may deliver copper to cuproenzymes located in the Golgi or secretion vesicles. In the immunofluorescence study, however, no evidence was provided to demonstrate that Cox17 antibody staining is specific or that the antibody staining is in the vicinity of cellular structures, such as ER and Golgi. That Cox17 is

highly expressed in certain neural cell lines and tissues is not surprising, nor does it necessarily imply that Cox17 is involved in brain and endocrine functions beyond a role in respiration. A number of other COX assembly factors, including *COX11*, *COX15*, *SCO1* (Petruzzella et al., 1998), and *SCO2* (Papadopoulou et al., 1999), show high levels of expression in brain. The dependence of neural tissues on aerobic respiration is underscored by the observation that brain is one of the most severely affected organs in mitochondrial disease (Di Donato, 2000). While additional functions for Cox17 cannot be ruled out, there is currently no substantial evidence to suggest that the primary role of mammalian COX17 is not in the COX assembly pathway.

The mitochondrial localization of human COX17 and its ability to functionally rescue a yeast *cox17* null mutant, suggests that mammalian COX17 plays a similar role in the cell as yeast Cox17p. The phenotype of mice carrying a homozygous disruption of *Cox17* also implies that mammalian COX17 is involved in mitochondrial copper metabolism (Takahashi et al., 2002). While mice heterozygous for the gene disruption are healthy and fertile, the homozygous disruption results in embryonic lethality between E8.5 and E10. Whole embryo sections taken at E6.5 show a severe reduction in COX activity, but not in the activity of other mitochondrial enzymes. Further evidence for a role in mitochondrial copper transport comes from the same study, in which Takahashi *et al.* demonstrate by an *in vitro* binding assay that Cox17 purified from bacteria is a copper-binding protein (Takahashi et al., 2002). The coordination of copper is ablated when the cysteinyl residues in KPCCXC, the conserved COX17 copper-binding motif, are mutagenized.

In the last few years, numerous studies have begun to investigate the role of COX17 in the mammalian cell. The present study suggests that human COX17 functions in COX assembly in a manner similar to its yeast counterpart. Many unanswered questions regarding COX17 function remain to be addressed, including the copper-binding abilities of the protein and the mechanism for COX17 mitochondrial import. Although no *COX17* mutations have been found in humans with mitochondrial disease to date (Horvath et al., 2000; Sacconi et al., 2003), the gene remains an attractive candidate as an underlying cause of human COX deficiency. Investigation in both mammals and yeast should continue to provide insight into the function of COX17, the putative mitochondrial copper chaperone.

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Chapter 5. Mutagenesis reveals a specific role for Cox17p in copper transport to cytochrome oxidase

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INTRODUCTION

Cytochrome oxidase (COX), the terminal electron acceptor of the mitochondrial respiratory chain, is an enzyme complex located in the mitochondrial inner membrane (Saraste, 1999). The mammalian COX enzyme is composed of 13 subunits (Tsukihara et al., 1996). The three catalytic core subunits (COX I to III) are encoded by the mitochondrial genome, with the remaining ten subunits encoded by nuclear DNA. In addition to the genes involved in COX subunit synthesis, studies in the yeast, *Saccharomyces cerevisiae*, have identified a number of genes that are required for various aspects of COX biogenesis, including heme A biosynthesis and copper provision (Tzagoloff and Dieckmann, 1990; Poyton and McEwen, 1996). Both the heme A moieties and copper atoms found in COX are critical for enzyme function, as both species play a direct role in electron transfer. The Cu_A centre on COX II, which contains two atoms of copper, is the first to receive electrons from the mobile electron carrier, cytochrome *c*. From the Cu_A site, electrons are transferred to heme a on COX I, then to the bimetallic heme a_3-Cu_B centre, at the active site of the enzyme (Tsukihara et al., 1995). To date, loss-of-function or null mutations in all of the genes associated with COX prosthetic group biosynthesis or insertion result in a respiration-deficient phenotype in yeast. The critical role of assembly factors involved in the procurement of prosthetic groups in COX biogenesis is underscored by the identification of lesions in the human homologues of a number of these genes, which have been demonstrated to be the underlying cause of some human COX deficiencies (Papadopoulou et al., 1999; Valnot et al., 2000a; Valnot et al., 2000b; Antonicka et al., 2003).

Three copper-binding proteins, Cox17p (Beers et al., 1997; Srinivasan et al., 1998; Heaton et al., 2000), Sco1p (Nittis et al., 2001; Beers et al., 2002), and Cox11p (Carr et al., 2002), have been shown to play a role in the provision of copper to the COX holoenzyme in yeast. Cox17p, a small, hydrophilic molecule, is believed to act as a copper chaperone, shuttling copper from the cytosol to the mitochondrial intermembrane space (Beers et al., 1997). The involvement of Cox17p in mitochondrial copper transport was originally proposed on the basis that supplemental copper in the growth medium could rescue the respiration-deficient phenotype of a *cox17* mutant (Glerum et al., 1996a). Because Sco1p was found to be a multicopy suppressor of a *cox17* mutant, it was hypothesized that Sco1p acts downstream of Cox17p in the mitochondrial copper transport pathway (Glerum et al., 1996b). A recent investigation of yeast *sco1* mutants suggests that Sco1p specifically provides copper to the Cu_A site on COX II (Dickinson et al., 2000). Studies in the bacterium, *Rhodobacter sphaeroides*, indicate that Cox11p functions to supply copper to the second copper centre, Cu_B, located on COX I (Hiser et al., 2000).

Currently, there is very little structural information for Cox17p and many of the unique features of the protein remain to be elucidated. As Cox17p lacks any previously identified mitochondrial targeting signal, the mechanisms that mediate Cox17p mitochondrial targeting and uptake are still unknown. In addition, it is unclear as to whether Cox17p enters the mitochondrion with bound copper. The presence of bound copper would suggest that Cox17p retains some degree of secondary structure as it crosses the outer membrane, which is contrary to our current understanding of mitochondrial import mechanisms in which proteins are unfolded before uptake.

Although there are a few proteins that are found in both the cytoplasm and the mitochondrion (for example, adenylate kinase (Schricker et al., 2002)), Cox17p is the only molecule that acts as a putative shuttle between the two subcellular compartments.

Taking advantage of the small size of Cox17p, a combination of site-directed and random mutagenesis was used in this study to examine the structure-function relationships in Cox17p. Only a small number of amino acid mutations affected Cox17p function and resulted in a detectable respiration-deficient phenotype. A subset of these respiration-deficient *cox17* mutants were found to have misassembled COX and a specific lack of Cox2p. This suggests that Cox17p acts upstream of Sco1p in the delivery of copper to COX II and predicts the existence of a subunit I-specific copper chaperone.

RESULTS

Cox17p Function Appears to be Insensitive to Mutation

COX17 proteins have been identified in virtually all eucaryotes studied to date. With the exception of the mammalian metallothionein, COX17 proteins do not share homology with any known proteins (Amaravadi et al., 1997). Although the COX17 and metallothionein proteins are similar in the sense that they are low molecular weight polypeptides with high cysteine content, the two proteins clearly function in different ways. The mammalian metallothioneins coordinate 12 atoms of copper (Dameron and Harrison, 1998), while Cox17p binds two (Beers et al., 1997; Srinivasan et al., 1998) or three (Heaton et al., 2000) copper atoms per molecule of protein. Copper is tightly bound to metallothionein, whereas copper is easily chelated from Cox17p (Beers et al., 1997; Srinivasan et al., 1998). Unlike Cox17p, for which very little structural information exists, the structure of metallothionein has been extensively studied (Kille et al., 1994).

To gain a better understanding of how Cox17p functions as a copper chaperone, both site-directed and random mutagenesis were employed to identify critical amino acids in Cox17p. Each *cox17* mutant was identified through automated sequencing, subcloned into a CEN plasmid, and transformed into a *cox17* null mutant. Transformants were tested to ensure cosegregation of the uracil prototrophy and the respiration phenotype. In addition, respiration-deficient mutants were mated with rho^0 tester strains to verify that mutations in mitochondrial DNA were not responsible for the reduced or absent growth on nonfermentable carbon sources.

In total, 74 point mutations were introduced into the *COX17* gene (Figure 5-1). Although the majority of mutations resulted in nonconservative amino acid substitutions, seven mutations led to conservative substitutions. Approximately 73% of the amino acids in Cox17p were altered (50 of 69), including 81% of the amino acids that are identical between the yeast and human COX17 proteins (17 of 21). Of the 74 mutations in *COX17*, 14 led to a discernible respiration-deficient phenotype (Table 5-1). Two of the 14 mutations that resulted in COX deficiency introduced a stop codon, prematurely truncating Cox17p. The remaining 12 mutations that caused a discernible phenotype were distributed over eight amino acids, seven of which are conserved between the yeast and human COX17 proteins.

From an examination of the amino acid sequence of Cox17p, it is apparent the protein contains a high proportion of charged amino acid residues. Two charged amino acids, glutamate and lysine, are particularly abundant, comprising 35% of the total amino acid content. To determine whether the glutamate and lysine residues are essential for Cox17p function, all of the conserved glutamate and lysine residues were mutated to

1	2	3	4	5	6	7	8	9	10	11	12
M	T	E	T	D	K	K	Q	E	Q	E	N
	T2A	E3G		D5A		K7I	Q8R	E9G		E11G	N12S
						K7E					

13	14	15	16	17	18	19	20	21	22	23	24
H	A	E	C	E	D	K	P	K	P	C	C
H13R	A14S	E15G	C16R	E17G		K19E		K21A	P22A		C24R
	A14T	E15V									

25	26	27	28	29	30	31	32	33	34	35	36
V	C	K	P	E	K	E	E	R	D	T	C
V25D	C26R			E29G	K30A	E31K	E32G	R33W	D34V	T35I	C36S
V25A				E29V	K30E			R33A	D34A		
V25T								R33D	D34R		

37	38	39	40	41	42	43	44	45	46	47	48
I	L	F	N	G	Q	D	S	E	K	C	K
I37V		F39S	N40Y	G41*	Q42L	D43G		E45G	K46R	C47R	K48R
I37T					Q42R	D43N		E45A	K46N		K48M
								E45V			K48E
								E45K			

49	50	51	52	53	54	55	56	57	58	59	60
E	F	I	E	K	Y	K	E	C	M	K	G
E49D	F50S		E52A	K53E		K55A	E56A	C57Y	M58A	K59*	
E49G				K53R						K59E	

61	62	63	64	65	66	67	68	69
Y	G	F	E	V	P	S	A	N
Y61F		F63I	E64D					N69I
								N69D

Figure 5-1. The amino acid sequence of Cox17p, showing mutations introduced by site-directed and random mutagenesis. Mutations shown in purple result in a respiration-deficient phenotype. Mutations shown in blue do not lead to a phenotype. Amino acids conserved between yeast and human COX17 proteins are shown in green. The copper-binding motif is indicated by a black bracket above the sequence. The asterisks indicate the introduction of a stop codon.

Table 5-1. Growth phenotype of respiration deficient *cox17* mutants.

Mutation[a]	Growth[b]	Mutation	Growth
C24R	-	D34V	1/4
V25D	1/2	D34R	-
C26R	-	C36S	1/2
R33D	-	G41*[c]	-
R33A	1/2	C47R	-
D34A	1/2	C57Y	-

[a]Mutant strains are designated by the Cox17p amino acid substitution they express.

[b] Growth refers to growth rate of the mutant strain on ethanol/glycerol, when compared with that of the wild-type strain.

[c]The asterisk denotes a stop codon.

alanine by site-directed mutagenesis. However, none of these alterations resulted in a respiration-deficient phenotype.

KPCCXC (where X is any amino acid), the copper-binding motif of Cox17p, is highly conserved between the yeast and mammalian COX17 proteins. Although the cysteine residues were mutagenized in an earlier study (Heaton et al., 2000), the functional significance of the lysine and proline residues in the copper-binding motif was unknown. Using site-directed mutagenesis, mutations were introduced at both amino acid residues. The growth of the K21A and P22A strains on nonfermentable carbon sources was not visibly different from a yeast strain expressing wild-type *COX17*, suggesting that neither alteration had any effect.

In addition to the three essential cysteines in the copper-binding motif, four cysteinyl residues, Cys16, 36, 47, and 57 are distributed throughout Cox17p. A nonconserved cysteine at position 16 was mutated to arginine, with no effect, indicating that this residue is dispensable for respiratory function. In contrast to an earlier report in which a C47S mutant was shown to be respiratory competent (Heaton et al., 2000), a C47R mutant was completely respiration deficient. Similar to C47R, a C57Y mutant strain failed to grow, while a C36S mutant grew at approximately half the wild-type rate.

In wild-type Cox17p, two conserved amino acids, arginine and aspartate, are found at residues 33 and 34, respectively. When mutated at either position, a respiration-deficient phenotype results. The introduction of an alanine at either residue retards respiratory growth, such that the R33A or D34A mutant yeast strains grow at half the rate of a wild-type strain. A D34V mutant strain shows a somewhat more severe phenotype, with growth occurring at one-quarter of the rate of a strain expressing wild-type *COX17*.

When either Arg33 or Asp34 are altered such that two positively- or negatively-charged amino acid residues occur at positions 33 and 34, respiratory growth is ablated. Both R33D and D34R mutant strains fail to grow on nonfermentable carbon sources, suggesting that the juxtaposition of oppositely-charged amino acids at residues 33 and 34 is important for Cox17p function.

A stop codon introduced at amino acid residue 41 was one of the two nonsense mutations generated by random mutagenesis. The protein truncation occurring at residue 40 was quite interesting from the perspective that in single copy, the mutant failed to grow on ethanol/glycerol medium. When present in multicopy, however, the mutant was able to grow at approximately one-third the rate of a yeast strain expressing wild-type *COX17*. In an attempt to examine the distribution of the G41* mutant protein in the yeast cell, fusion proteins were made, consisting of an in-frame FLAG epitope fused to either the N-terminus or the C-terminus of the mutant Cox17p. Epitope-tagged proteins were also made with the K59* truncated mutant, which consisted of the FLAG epitope fused in-frame to the N-terminus of the mutant Cox17p and the Xpress epitope fused in-frame to the C-terminus of the mutant Cox17p. When expressed in the *cox17* null mutant yeast strain, none of the four fusion proteins could be detected with antibody against the epitope tags. These results suggest that when present on mutant Cox17p, the FLAG and Xpress epitopes are inaccessible to the antibody or are degraded. Alternatively, the mutant fusion proteins are not expressed at detectable levels. When the FLAG epitope is present at the C-terminus of wild-type Cox17p, a band representing the fusion protein can be detected with the anti-FLAG antibody. An N-terminal FLAG epitope on wild-type

Cox17p is barely detectable, suggesting that an N-terminal tag may not be well-tolerated by the protein (Figure 5-2).

The majority of the *cox17* mutants that exhibited a respiration-deficient phenotype were investigated in both the 'a' and 'α' mating type *cox17* null mutant strains. Although there should be no difference in the respiratory growth of the mutants in the two Δ*cox17* strains, a different growth phenotype was noted in two of the mutants. While the R33W mutant grew at one-half of the wild-type rate in the 'a' mating type strain, it grew at one-third of the wild-type rate in the 'α' mating type strain. Similarly, a K59* mutant did not grow on nonfermentable carbon sources when transformed into the 'α' mating type, but showed one-third of wild-type growth when expressed in the 'a' strain. To determine if there was a detectable difference between the strains, the auxotrophies and the respiratory growth of the two *cox17* null mutant strains were tested simultaneoulsy. As there was no difference in the behaviour of the two strains, it is unclear as to why the two mutants showed disparate growth in the two *cox17* null mutant strains.

In the general case, the respiratory deficiency of a mutant yeast strain shows a correlation with the amount of residual COX activity in the strain. To investigate the COX activity of the mutants generated in this study, mitochondria were isolated from each strain and assayed for COX activity (Figure 5-3). Mutants that displayed slower than wild-type growth on nonfermentable carbon sources, including V25D, R33A, R33W, D34A, and D34V, exhibited reduced COX activity relative to wild-type yeast. Mutants that did not grow on ethanol/glycerol, such as R33D, D34R, G41*, C47R, and C57Y, showed COX activity levels similar to the *cox17* null mutant strain.

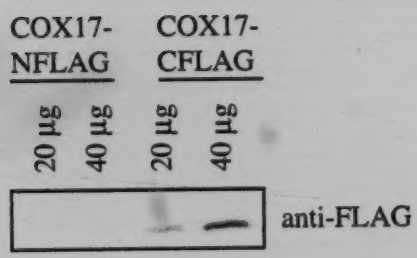

Figure 5-2. Expression of yeast Cox17p-FLAG fusion proteins. Twenty and 40 µg of yeast whole cell lysate protein were separated in 15% polyacrylamide gels containing glycerol and tranferred to nitrocellulose, followed by probing with antibody against the FLAG epitope (anti-FLAG). COX17-NFLAG = yeast Cox17p with in-frame N-terminal FLAG epitope expressed from YEp352; COX17-CFLAG = yeast Cox17p with in-frame C-terminal FLAG epitope expressed from YEp352.

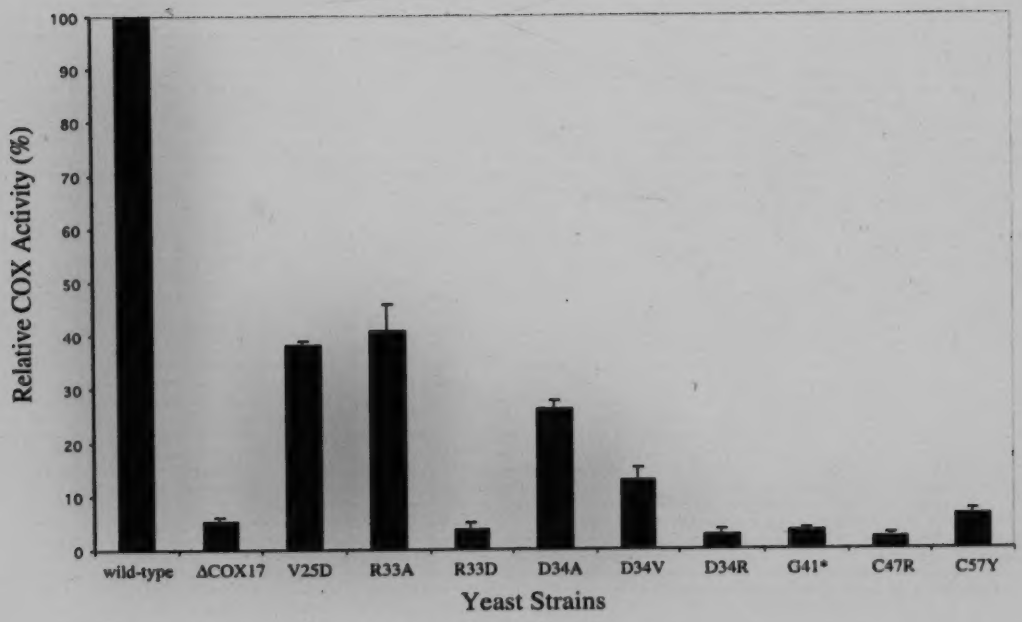

Figure 5-3. COX activity in respiration-deficient *cox17* mutants. COX activity was measured spectrophotometrically using mitochondria from *cox17* null mutant strains expressing mutated Cox17p. Activities are expressed as a percentage of wild-type COX activity and are the average of four assays. The mutants are designated by their mutation. Lines above the bars represent the standard error of the mean. ΔCOX17 = *cox17* null mutant.

Some *cox17* Mutants have Misassembled Cytochrome Oxidase

In most COX assembly mutants studied to date, the respiratory phenotype and residual COX activity of the yeast strain are found to correlate with the amount of assembled COX holoenzyme. The degree to which COX assembly has occurred can be gauged from the size of the peak at 603 nm in mitochondrial cytochrome spectra, which is due to the absorbance of the COX *a*-type cytochromes (*a* and a_3). COX assembly factor mutants that are unable to grow on nonfermentable carbon sources and have no COX activity do not show a detectable peak at 603 nm, indicating that the COX enzyme complex has not been assembled. The recent report of a *sco1* S240F mutant represents an exception to this generalization (Dickinson et al., 2000). Although the S240F mutant was respiration deficient and showed a severe reduction in COX activity, the strain exhibited a discernible aa_3 peak. The peak, however, was shifted by approximately 5 nm to the blue, suggesting the presence of a misassembled COX enzyme.

To examine the extent of COX assembly in the *cox17* mutants generated in this study, mitochondrial cytochromes were extracted from the various mutant strains and analyzed spectrophotometrically (Figures 5-4 and 5-5). Spectral analysis indicated that the *cox17* mutants fall into three categories. Several mutants, including V25D, R33A, R33W, D34A, and D34V, exhibit a reduced cytochrome aa_3 peak consistent with retarded growth on ethanol/glycerol medium and partially reduced COX activity. Two mutants, R33D and G41*, which do not grow on nonfermentable carbon sources and do not show any residual COX activity, lacked a discernible cytochrome aa_3 peak. The absence of a cytochrome aa_3 peak in these mutants suggests that there is no assembled COX enzyme present. The remaining mutants, D34R and C47R, which did not grow on

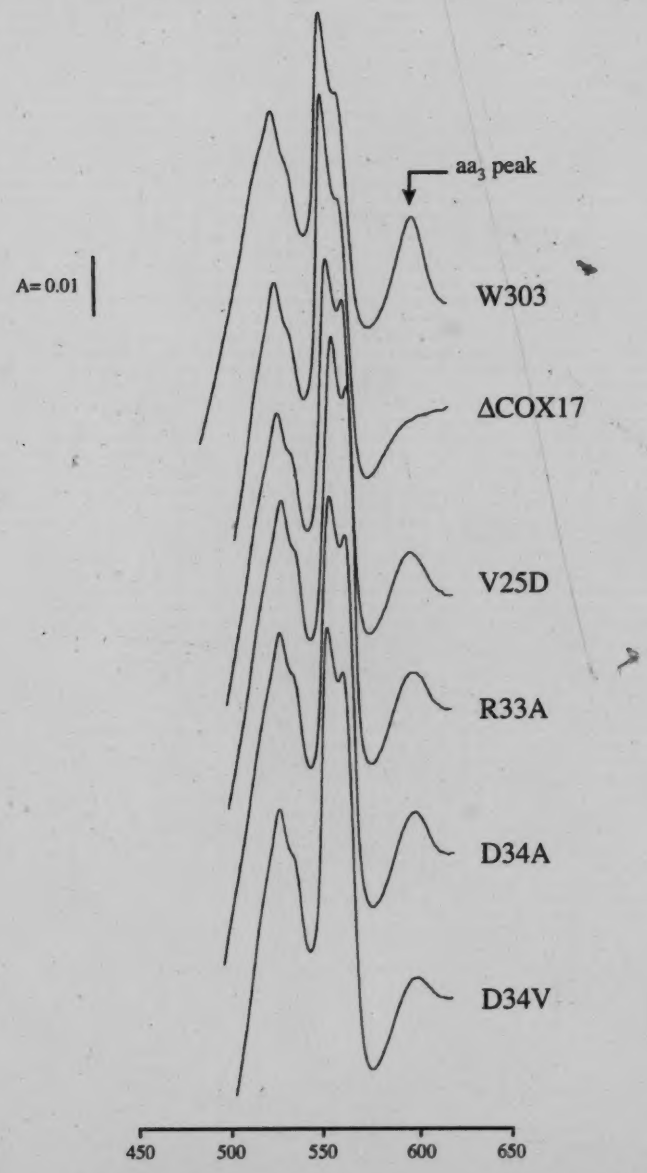

Figure 5-4. Cytochrome spectra of yeast *cox17* mutants V25D, R33A, D34A, and D34V. All of the mutants show partial respiratory growth on nonfermentable carbon sources. Cytochrome spectra were obtained from mitochondria extracted and solubilized with 5% potassium chloride and 1% deoxycholate at a protein concentration of 6.5 mg/mL. Difference spectra of extracts reduced with sodium dithionite and oxidized with potassium ferricyanide were obtained at room temperature. The α band corresponding to cytochrome aa_3 has a maximum at 603 nm and is indicated by an arrow. W303 = wild-type yeast; ΔCOX17 = *cox17* null mutant strain.

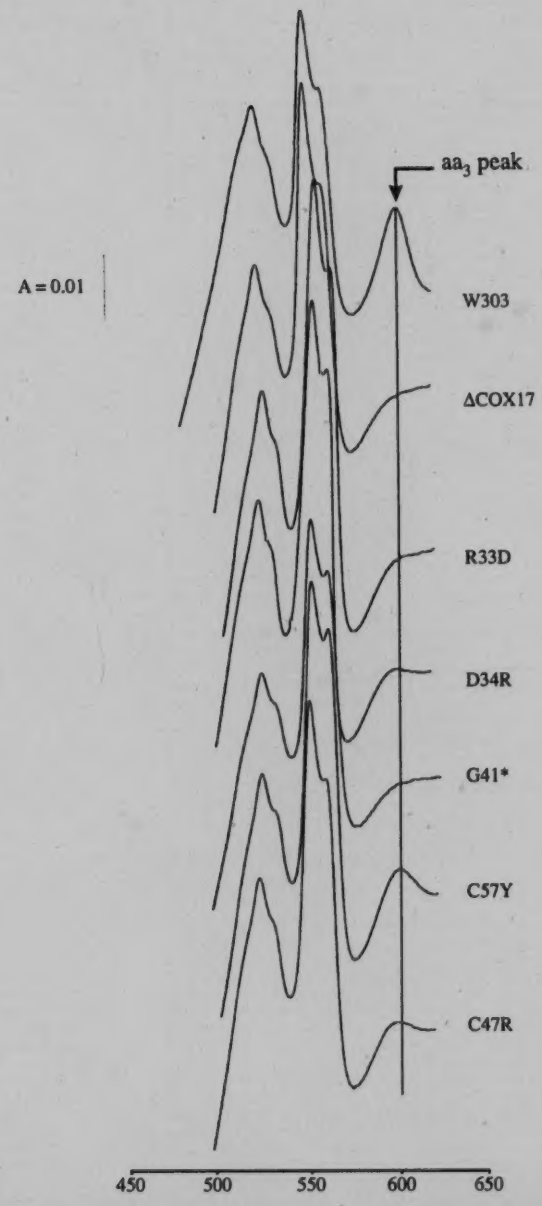

Figure 5-5. Cytochrome spectra of yeast *cox17* mutants R33D, D34R, G41*, C57Y, and C47R. None of the mutants show respiratory growth on nonfermentable carbon sources. Cytochrome spectra were obtained from mitochondria extracted and solubilized with 5% potassium chloride and 1% deoxycholate at a protein concentration of 6.5 mg/mL. Difference spectra of extracts reduced with sodium dithionite and oxidized with potassium ferricyanide were obtained at room temperature. The α band corresponding to cytochrome aa_3 has a maximum at 603 nm and is indicated by an arrow. W303 = wild-type yeast; ΔCOX17 = *cox17* null mutant strain.

nonfermentable carbon sources or have detectable COX activity, did exhibit a small cytochrome aa_3 peak. The peaks in these two mutants, however, appeared to be shifted approximately 2 nm to the blue, suggesting that the two heme moieties are present in an abnormal environment and that the small amount of COX enzyme present is misassembled.

Some *cox17* Mutants Display a Selective Loss of Subunit II

In general, COX assembly in yeast is thought to be an all-or-nothing phenomenon. This belief is largely due to the observation that in many of the COX assembly mutants studied thus far, the mitochondrial DNA-encoded COX subunits are rapidly degraded in the absence of assembled enzyme. The *sco1* S240F mutant has recently been shown to be an exception (Dickinson et al., 2000). In addition to the shifted cytochrome aa_3 signal, the mutant shows a specific loss of COX subunit II. The levels of both COX I and the nuclear-encoded subunits are at or near wild-type levels.

To investigate the steady-state levels of mitochondrial DNA- and nuclear DNA-encoded COX subunits in the *cox17* mutants, mitochondrial proteins from the various mutant strains were separated by SDS-PAGE, transferred to nitrocellulose membrane, and probed with antibodies against the COX subunits (Figures 5-6, 5-7, and 5-8). While the nuclear DNA-encoded subunits in the *cox17* null mutant are present at levels found in wild-type yeast, the strain shows a severe reduction in COX I and an almost complete absence of COX II and COX III. In this study, the strains showing slower growth on nonfermentable carbon sources relative to wild-type yeast, including V25D, R33A, R33W, D34A, and D34V, had somewhat reduced levels of all three mitochondrial DNA-encoded COX subunits. Interestingly, however, mutants that do not show any growth on

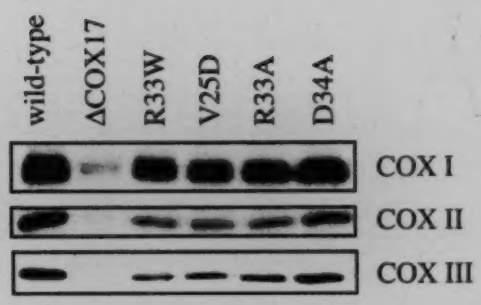

Figure 5-6. Mitochondrial DNA-encoded cytochrome oxidase subunit levels in yeast *cox17* R33W, V25D, R33A, and D34A mutants. All of the mutants show partial respiratory growth on nonfermentable carbon sources. Ten μg of mitochondrial protein were separated on a 12% polyacrylamide gel and transferred to nitrocellulose membrane, followed by probing with antibodies against subunit I (COX I), subunit II (COX II) and subunit III (COX III).

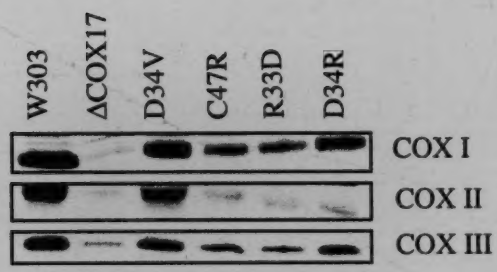

Figure 5-7. Cytochrome oxidase subunits I, II, and III in yeast *cox17* D34V, C47R, R33D, and D34R mutants. Ten μg of mitochondrial protein were separated on a 12% polyacrylamide gel and transferred to nitrocellulose membrane, followed by probing with antibodies against subunit I (COX I), subunit II (COX II), and subunit III (COX III).

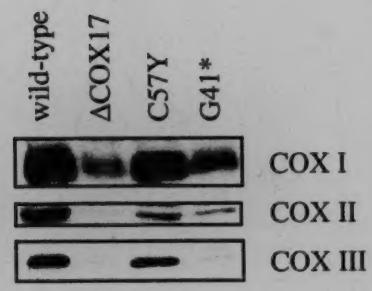

Figure 5-8. Cytochrome oxidase subunits I, II, and III in yeast *cox17* C57Y and G41* mutants. Ten μg of mitochondrial protein were separated on a 12% polyacrylamide gel and transferred to nitrocellulose membrane, followed by probing with antibodies against subunit I (COX I), subunit II (COX II) and subunit III (COX III).

ethanol/glycerol medium (R33D, D34R, G41*, C47R) have only somewhat reduced levels of COX I, with a more marked reduction in COXIII, and an almost complete loss of COX II. As in the *cox17* null mutant, nuclear DNA-encoded COX subunits appeared to be at or near wild-type levels in all of the *cox17* point mutants (Figure 5-9).

Some Mutants are Impaired in Mitochondrial Uptake

In the yeast cell, Cox17p is partitioned between the cytosol and the mitochondrial intermembrane space. Approximately 60% of the protein is found in the mitochondrion, with the remaining 40% of Cox17p localized to the cytoplasm (Beers et al., 1997). To determine if the steady-state levels of Cox17p are affected in the *cox17* mutants, mitochondrial and post-mitochondrial supernatant fractions from the mutant strains were probed with antibody against Cox17p (Figures 5-10, 5-11, and 5-12). With the exception of the V25D mutant, all strains exhibited a decrease in the steady-state levels of Cox17p relative to the wild-type yeast strain. The decrease in Cox17p was most pronounced in the strains that are not capable of respiratory growth (Figures 5-11 and 5-12). Four nonrespiring strains had severely reduced levels of Cox17p in the mitochondrial fraction. In particular, the C47R mutant had no detectable Cox17p in the mitochondrial fraction, although the protein could be detected in the post-mitochondrial supernatant fraction, which contains the cytosolic components. The reduction or absence of Cox17p in the mitochondrial fraction of nonrespiring mutants may indicate mutated Cox17p has greater difficulty entering the mitochondrial intermembrane space.

In the case of several mutant strains, the migration of mutant Cox17p in SDS-PAGE gels appeared to be altered. Cox17p carrying a V25D mutation seemed to migrate more slowly than wild-type Cox17p, while a protein with an R33W mutation appeared to

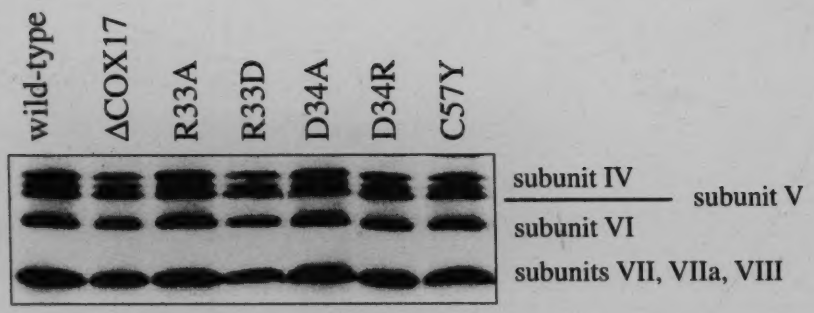

Figure 5-9. Nuclear DNA-encoded cytochrome oxidase subunits in *cox17* mutants. Ten μg of mitochondrial protein were separated on a 16.5% polyacrylamide, 6 M urea gel and transferred to nitrocellulose membrane. The blot was probed with an antibody that recognizes some of the subunits (subunits IV, V, VI, VII, VIIa, and VIII) encoded by the nuclear genome.

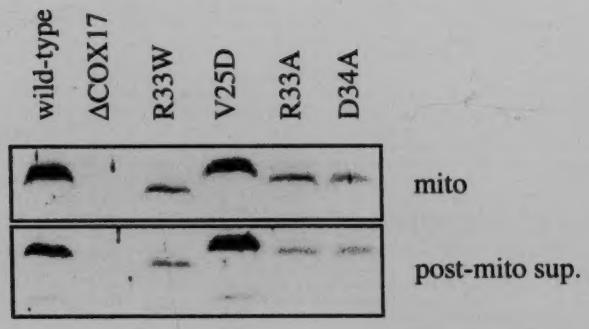

Figure 5-10. Expression of Cox17p in yeast *cox17* mutants R33W, V25D, R33A, and D34A. All of the mutant strains show partial respiratory growth on nonfermentable carbon sources. Twenty μg of mitochondrial protein (mito) and 30 μg of post-mitochondrial supernatant protein (post-mito sup.) were separated on a 15% polyacrylamide gel containing glycerol and transferred to nitrocellulose membrane, followed by probing with antibody against yeast Cox17p.

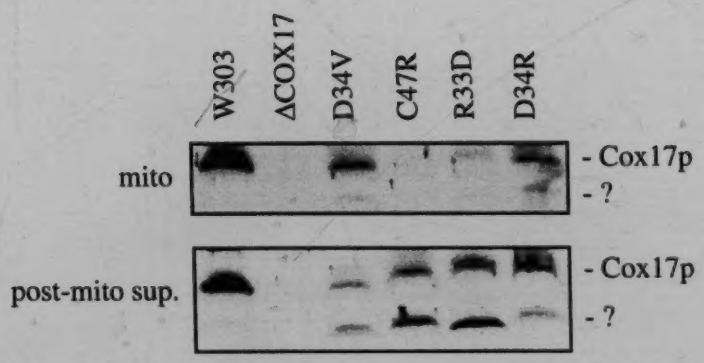

Figure 5-11. Expression of Cox17p in yeast *cox17* D34V, C47R, R33D, and D34R mutants. Twenty μg of mitochondrial protein (mito) and 30 μg of post-mitochondrial supernatant protein (post-mito sup.) were separated on a 15% polyacrylamide gel containing glycerol and transferred to nitrocellulose membrane, followed by probing with antibody against yeast Cox17p.

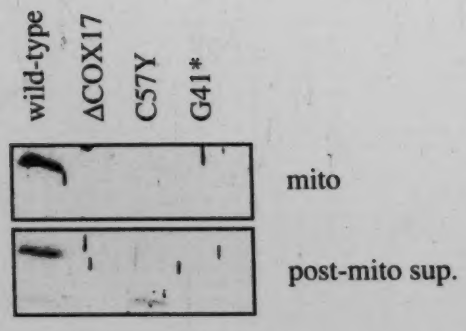

Figure 5-12. Expression of Cox17p in yeast *cox17* mutants C57Y and G41*. Neither of the mutant strains shows any growth on nonfermentable carbon sources. Twenty μg of mitochondrial protein (mito) and 30 μg of post-mitochondrial supernatant protein (post-mito sup.) were separated on a 15% polyacrylamide gel containing glycerol and transferred to nitrocellulose membrane, followed by probing with antibody against yeast Cox17p. As the Cox17p antibody is directed against the C-terminus of the protein, no Cox17p band would be expected in the lanes for the G41* mutant.

migrate more quickly than wild-type Cox17p (Figure 5-10). The difference in the rates of migration relative to wild-type Cox17p suggests that the difference in charge or structure that results from the introduced mutations affects the behaviour of Cox17p during SDS-PAGE.

The majority of the mutants appear to accumulate a lower molecular weight band, which may represent a Cox17p degradation product. This polypeptide is clearly recognized by the Cox17p antibody, which is directed against the C-terminus of the protein. The putative degradation product is most prevalent in the post-mitochondrial supernatant fractions.

DISCUSSION

Cox17p function appears to be insensitive to mutation, which may reflect an intrinsic property of this protein. Analysis of the amino acid sequence of Cox17p suggests that the protein may be "natively unfolded". At the amino acid level, natively unfolded proteins are characterized by a large net charge at neutral pH, resulting from non-neutral pI values, in addition to a low content of hydrophobic amino acids (Uversky, 2002). Typically, intrinsically disordered proteins are depleted in Ile, Leu, Val, Trp, Tyr, Cys, and Asn. The hydrophobic amino acids belonging to this group are believed to impart order to a protein. In addition, natively unfolded proteins tend to be enriched in Glu, Lys, Arg, Gly, Gln, Ser, Pro, and Ala, in which the charged or polar amino acids contribute disorder to a protein (Denning et al., 2002). When comparing the amino acid sequence of Cox17p to the *S. cerevisiae* proteome (Figure 5-13), Cox17p contains 29% ordering amino acids, less than the 38% found in the *S. cerevisiae* proteome. Approximately 57% of the amino acids in Cox17p contribute to disorder, while only 46%

	A	R	N	D	C	Q	E
***S. cerevisiae* proteome**	5.50	4.44	6.06	5.78	1.31	3.89	6.51
Cox17p	2.90	1.45	4.35	5.80	10.14	4.35	18.84

	G	H	I	L	K	M	F
***S. cerevisiae* proteome**	4.99	2.13	6.56	9.62	7.3	2.09	4.55
Cox17p	4.35	1.45	1.45	1.45	15.94	2.90	4.35

	P	S	T	W	Y	V
***S. cerevisiae* proteome**	4.32	9.03	5.82	1.05	3.33	5.61
Cox17p	5.80	2.90	4.35	5.80	2.90	2.90

	Order	Disorder
***S. cerevisiae* proteome**	38.09	45.98
Cox17p	28.99	56.52

Figure 5-13. A comparison of the amino acid compositions (% amino acid frequency) of Cox17p *versus* the *S. cerevisiae* proteome. The order conferring amino acids (N, C, I, L, F, W, Y, and V) are generally depleted in natively unfolded proteins and are highlighted in green. The disorder conferring amino acids (A, R, Q, E, G, K, P, and S) are generally enriched in natively unfolded proteins and are highlighted in blue. After Denning *et al.*, 2002.

of amino acids in the yeast proteome facilitate disorder. The percentage of ordering and disordering amino acids in Cox17p (Figure 5-14) are similar to those found in the nucleoporin, Nup2p, which is believed to be a natively unfolded protein (Denning et al., 2002). Nup2p plays a role in nuclear trafficking and shuttles between the cytoplasm and the nucleus, a situation that may be analogous to the proposed movement of Cox17p between the cytoplasm and the mitochondrial intermembrane space. Although substantial biophysical characterization is required to confirm the structural nature of Cox17p, a flexible or less ordered secondary structure may explain how Cox17 can retain function in the presence of many different nonconservative amino acid substitutions.

In this study, it was found that a C47R *cox17* mutant was unable to grow on nonfermentable carbon sources, contrasting a previous report that found a C47S mutant was respiratory competent (Heaton et al., 2000). The respiration-deficient phenotype of the C47R mutant suggests that the nature of residue 47 is important for Cox17p and that the presence of a bulky, charged amino acid at position 47 ablates Cox17p function. Because cysteine and serine are polar, uncharged, and sterically similar, it seems likely that the two amino acids can be interchanged at this position without disrupting the integrity of Cox17p.

A number of amino acid replacements were introduced into Cox17p at residues Arg33 and Asp34, all of which resulted in a COX-deficient phenotype. Although the introduction of an uncharged amino acid at either position only slowed respiratory growth relative to yeast expressing wild-type *COX17*, the presence of similarly charged amino acids (either both positive or both negative) at the two positions completely abolished respiratory growth. These results suggest that the juxtaposition of a positively-charged

M T E T D K K Q E Q E N

H A E C E D K P K P C C

V C K P E K E E R D T C

I L P N G Q D S E K C K

E F I E K Y K E C M K G

Y G F E V P S A N

Figure 5-14. The amino acid sequence of Cox17p. Amino acids that confer order to the protein (N, C, I, L, F, W, Y, and V) are shown in red. Amino acids that confer disorder to the protein (A, R, Q, E, G, K, P, and S) are shown in blue.

and a negatively-charged amino acid at residues 33 and 34 is important for the maintenance of Cox17p function.

The mutants described in this study fall into three categories. In the first set of mutants, the phenotype is characterized by slower respiratory growth, a reduction in COX activity, a decrease in assembled COX holoenzyme, and somewhat decreased steady-state levels of the mitochondrial DNA-encoded COX subunits. In the second category, the mutants are incapable of respiratory growth and show an absence of COX activity and assembly, similar to the *cox17* null mutant. The third group of mutants also are unable to grow on nonfermentable carbon sources and have no COX activity, but do show some indication of COX assembly. In all of the nonrespiring mutants, the levels of COX I are somewhat decreased, but not to the same extent as COX II, which is almost completely absent in the mutants. The specific loss of COX II, which is reminiscent of the *sco1* S240F mutant, may suggest that Cox17p is providing copper to the Cu_A site and is upstream of Sco1p. The phenotype of the *cox17* mutants with respect to the loss of mitochondrial DNA-encoded subunits may not be as distinct as the *sco1* S240F mutant, given that Cox17p acts upstream of Sco1p. If the block in COX II copper provision occurs at Cox17p, rather than Sco1p, there may be more opportunity for the proteolytic degradation of all mitochondrial DNA-encoded COX subunits, rather than just COX II.

The significance of the reduction of COX III in the majority of the *cox17* mutants is currently unclear. This decrease in COX III is also apparent in the *sco1* S240F mutant strain (Figure 5-15). In previous studies of COX assembly factor mutants, the steady-state levels of COX III, which does not contain any prosthetic groups, have not been assessed. In an investigation of COX assembly in cultured human cells, it was suggested

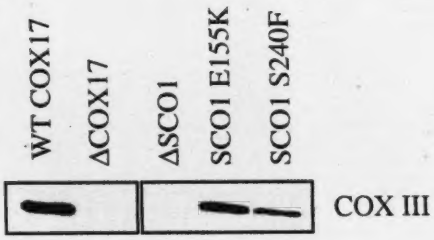

Figure 5-15. Subunit III levels in *sco1* mutants. Subunit III levels are reduced in the yeast *sco1* S240F mutant, which is respiration deficient, relative to the levels in the *sco1* E155K mutant, which is respiration competent. The two panels are taken from the same Western blot, although intervening lanes have been removed. Ten μg of mitochondrial protein were separated on a 12% polyacrylamide gel and transferred to nitrocellulose membrane, followed by probing with antibodies against subunit III (COX III). WT COX17 = wild-type *COX17* expressed from YCplac33 in a *cox17* null mutant; ΔCOX17 = *cox17* null mutant; ΔSCO1 = *sco1* null mutant; SCO1 E155K = *sco1* E155K mutant; SCO1 S240F mutant = *sco1* S240F mutant.

that COX II and COX III are incorporated into the COX enzyme complex at the same step in the assembly pathway (Nijtmans et al., 1998). Therefore, it is possible that the absence or reduction of COX II affects the insertion or stability of COX III. Further studies of COX III in the context of COX assembly factor mutants are needed to gain additional insight into the relationship between COX II and COX III during COX assembly.

In the COX assembly model mentioned above (Nijtmans et al., 1998), it was proposed that COX I, which contains bound copper at the Cu_B site, is the first subunit to be assembled into the COX enzyme complex. As seen in both the *sco1* S240F and *cox17* mutants, the specific loss of COX II suggests that copper addition to the Cu_A site occurs late in assembly. This provides a temporal separation in the requirements for Cox11p and for Sco1p/Cox17p at the two copper sites during assembly. Since Cox17p appears to play a role in copper delivery specifically to Cox2p, it suggests that there may be another copper chaperone that provides copper to COX I. Cox19p is a recently identified COX assembly factor with a weak homology to Cox17p, characterized by a conservation of the spatial distribution of the cysteine residues (Nobrega et al., 2002). As Cox17p and Cox19p share a similar subcellular distribution, Cox19p may be a candidate copper chaperone.

In addition to suggesting that Cox17p may serve as a copper chaperone specific for subunit II, the mutants presented here may help shed some light on the process of mitochondrial copper uptake of Cox17p. Most of the mutants exhibit decreased steady-state levels of Cox17p, but still have a small pool of detectable protein in the mitochondrial fraction. Only two mutants, C47R and C57Y, appear to lack

mitochondrially-localized Cox17p. However, on very long exposures of Western blots, a small amount of Cox17p is detected in mitochondria from the C57Y strain. Because there is a small cytochrome aa_3 peak and only slightly reduced levels of subunit I in the C47R mutant, it seems likely that there is a small, but undetectable amount of Cox17p present in the mitochondria. Previous experiments have shown that a Cox17p fusion protein that appeared to localize exclusively to the cytoplasm was able to rescue the respiration deficiency of the *cox17* null mutant (Glerum et al., 1996a), suggesting that even undetectable amounts of Cox17p are adequate for COX assembly. In addition, the finding that a mutant lacking the C-terminal one-third of Cox17p (G41*) can still confer some degree of respiratory competence when present at high copy number suggests that the C-terminus may not be essential for mitochondrial uptake of Cox17p.

The C57Y mutant appears to be an exception among the nonrespiring *cox17* mutants. Although this mutant does not grow on nonfermentable carbon sources and does not have any COX activity, the cytochrome spectrum shows a peak at 603 nm, indicating that COX assembly is occurring. Consistent with a phenotype in which some assembled COX is present, the C57Y mutant does not show as extreme a loss of the mitochondrial DNA-encoded subunits as the remainder of the nonrespiring mutants. Further investigation is required to explain the presence of a correctly assembled but completely inactive COX holoenzyme in this mutant.

The observation that Cox17p does not share homology with any other yeast protein suggests that this putative mitochondrial copper chaperone is a unique molecule. In this study, several amino acid residues that appear to be important for the structure and function of Cox17p have been identified. Further biochemical and biophysical

characterization of both wild-type and mutant proteins should lead to a greater understanding of Cox17p function. Additional analysis of the *cox17* mutants described here may help define the features of Cox17p that contribute to its function as a copper chaperone, including the properties that are involved in mitochondrial localization. Using the mutants that exhibit an altered distribution of Cox17p, yeast genetic screens may allow the identification of factors involved in mitochondrial uptake of Cox17p, the mechanism of which remains completely unknown.

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Chapter 6. Discussion

Human COX17

In this thesis, the deciphering of the human *COX17* gene structure is described. During the course of the investigation, I determined that two *COX17* loci exist in the human genome, one of which represents a processed pseudogene. Until this study of human *COX17*, it had been reported by two different consortia that the gene encoding the human *COX17* cDNA is localized to chromosome 13. Using radiation hybrid mapping, however, I have demonstrated that the pseudogene, which is extremely similar to the *COX17* cDNA, is found on chromosome 13. A similar approach was used to show that the expressed version of *COX17* lies on the long arm of chromosome 3. The clarification of the chromosomal location of *COX17*, in addition to the determination of its genomic structure, has allowed patients with isolated COX deficiency to be assessed for mutations in the *COX17* gene.

In the study of human *COX17*, I have also determined that the gene is ubiquitously expressed, although at varying levels in different tissues. The ubiquitous but varied nature of *COX17* expression is consistent with the expression of other COX assembly factors genes (Shoubridge, 2001), although it is currently unclear as to how the genes involved in COX biogenesis are regulated in different tissues. An analysis of the promoter regions in COX assembly factor genes, including *COX17*, may provide an indication of how gene expression is controlled and whether there is a common mechanism of regulation in these genes. With the completion of the Human Genome Project, a computer-based comparsion of regulatory elements in COX assembly factor genes can be carried out. A greater understanding of tissue-specific regulation has

important clinical relevance, as it may explain why some tissues are severely affected in a particular isolated COX deficiency, while other tissues are relatively spared, showing a relatively minor decrease in COX activity and no indication of a COX assembly defect. As an example, it remains to be seen why mutations in *SURF1* are primarily associated with neurodegeneration in the brain (Tiranti et al., 1998; Zhu et al., 1998), while mutations in *COX15* are an underlying cause of hypertrophic cardiomyopathy (Antonicka et al., 2003). Given that there are tissue-specific isoforms of some COX subunits, it is possible that different COX assembly pathways exist in different tissues or cell types. In addition, certain COX assembly factors may play a lesser role in COX assembly in a particular tissue or cell type, such that their reduced function or absence is less catastrophic than in a tissue where they play a more prominent role.

A number of groups, including our lab, have screened patients with isolated COX deficiency for mutations in human *COX17*. To date, however, no mutations have been found (Horvath et al., 2000; Sacconi et al., 2003). Interestingly, no lesions have been identified in the genes encoding ATOX1 or CCS, the other two cellular copper chaperones (Mercer, 2001; Moore et al., 2002). Recently, null mutant mice have been generated for each of the copper chaperone genes. While *Ccs* -/- mice are viable and show normal levels of Cu,Zn superoxide dismutase (SOD1), they show marked reductions in SOD1 activity, increased sensitivity to paraquat, and reduced female fertility (Wong et al., 2000). Mice deficient in Atox1 show failure to thrive immediately after birth, characteristic features of copper deficiency, elevated levels of intracellular copper, and increased perinatal lethality (Hamza et al., 2001). The complete absence of murine *Cox17* results in severely reduced levels of COX activity in the developing

embryo, with embryonic lethality occurring between E8.5 and E10 (Takahashi et al., 2002). Although it has been suggested that Cox17 is the copper chaperone acting downstream of Ctr1 during early embryogenesis (Takahashi et al., 2002), it seems more likely that the three copper chaperones are required at temporally different periods throughout life. In the case of each knock-out mouse, the ablation of a copper chaperone gene indicates that these metallochaperones play an important role in copper homeostasis in the mammalian cell. Despite the fact that mutations have not been identified in human patients, the phenotypes of the null mutant mice suggest that lesions in copper chaperone genes may be a cause of human disease. Continued efforts in the screening of patients may lead to the identification of individuals with mutations in metallochaperone genes, including *COX17*.

Although it was previously shown that human COX17 is not a mitochondrial protein (Kako et al., 2000), the data presented in chapter four of this thesis suggest that the protein does localize to the mitochondrion. By two separate methods, including immunoblotting and immunofluorescence, I have demonstrated that human COX17 is detected in mitochondria. These results suggest that human COX17 has a similar cellular distribution to that of its yeast counterpart. Using a functional complementation approach, I have shown that the presence of human COX17 in the heterologous environment of the yeast cell results in COX assembly and a significant increase in COX activity in the absence of yeast Cox17p. That human COX17 is capable of partially rescuing a *cox17* null mutant infers that the two proteins have a similar function in the eucaryotic cell. Based on the previous localization of murine Cox17 to perinuclear regions in the vicinity of the ER and Golgi, as well as the high levels of expresson in a

pituitary tumour cell line (Kako et al., 2000), it was originally proposed that mammalian COX17 is involved in brain and endocrine functions, in addition to having a role in cellular respiration. While additional roles for COX17 in the mammalian cell cannot be conclusively ruled out, the conservation of function between yeast and human COX17 proteins suggests that human COX17 is involved in mitochondrial copper transport, playing a role similar to that in the yeast cell. The conservation of other copper transport pathways between yeast and human cells, such as pathway in which copper is delivered to ATP7A and ATP7B (Ccc2p, in yeast) by ATOX1 (Atx1p, in yeast), also argues against the involvement of COX17 in the shuttling of copper to the secretory pathway.

Recent studies have shown that mouse Cox17 expressed and purified as a GST fusion protein in bacteria binds 2.5 ions of copper per molecule of protein (Takahashi et al., 2002). As in yeast Cox17p, mutagenesis of the three cysteines in the copper-binding motif abrogated copper binding in murine Cox17. Slightly less copper was bound to murine Cox17 when the protein was truncated at amino acid residue 48. Given the conservation of function between yeast and human COX17, and the demonstration that murine Cox17 is a copper-binding protein, it is likely that an investigation of purified human COX17 will show that the human protein also binds copper.

Many issues remain to be addressed with respect to COX17 function in higher eucaryotes. Although *COX17* expression has been studied by Northern blot analysis in multiple human, murine and rat tissues, as well as in a number of murine tumour cell lines (Kako et al., 2000), it is not clear whether COX17 expression is altered under different cellular conditions. An examination of *COX17* expression at the mRNA and protein level when copper is present in excess, or is depleted, could be used to determine

whether COX17 is affected by cellular copper levels. An investigation of COX17 expression in cell lines from patients with copper storage disorders, such as Wilson or Menkes disease, may shed light on whether COX17 expression or distribution is affected when defects in other copper transport pathways are present.

In the absence of naturally-occurring mutations in the human *COX17* gene, very little is known about the structure-function relationships in mammalian COX17. From an examination of the amino acid sequence of mammalian COX17 proteins, it is apparent that 25% of the first 20 N-terminal amino acid residues are proline. The reason for the high proportion of these residues is unknown. It is also unclear if the conserved distribution of the histidine residues in the C-terminal region of the protein is significant (Chen et al., 1997). To determine which amino acids, including the N-terminal proline and C-terminal histidine residues, play a critical role in COX17 function, mutations could be introduced into the human *COX17* cDNA and analyzed by expressing the mutant gene in a yeast *cox17* null mutant strain. A decrease in the growth rate of strains expressing a mutant cDNA relative to the rate of the wild-type cDNA on nonfermentable carbon sources would indicate that COX17 function is being compromised by the amino acid alterations. To identify the amino acids or regions of human COX17 that are involved in mitochondrial localization and uptake, mutant cDNAs bearing an epitope tag could be transiently or stably transfected into mammalian cells. A change in the cellular distribution of COX17 (e.g. the absence of mitochondrial localization), detected by either subcellular fractionation or immunfluorescence, would suggest that the introduction of a particular mutation has altered COX17 uptake. To identify essential amino acids in murine Cox17, mutant cDNAs could be expressed in the background of a *Cox17* -/- cell

line. Altered cDNAs that fail to restore COX activity or do not restore activity to the same extent as a normal copy of *Cox17* in the null mutant cell line, may help to define amino acid residues that are required for normal Cox17 function.

Yeast Cox17p

Although important information can be garnered from the investigation of mammalian COX assembly factors, studies in the yeast, *S. cerevisiae*, have been instrumental in advancing our understanding of COX biogenesis. For a number of reasons, including that it is a facultative anaerobe, and amenable to genetic and biochemical manipulation, yeast has proven to be a very useful model organism for the study of COX assembly. In addition, COX is structurally and functionally similar in yeast and humans, and the complete sequence of the yeast genome is known. The utility of the yeast studies is underscored by the fact that all of the COX assembly factors genes associated with human COX deficiency were first identified in yeast.

In chapter five of this thesis, a combination of random and site-directed mutagenesis was used to further examine structure-function relationships in the yeast COX assembly factor, Cox17p. Although a previous investigation had determined that the three cysteinyl residues in the copper-binding motif are essential (Heaton et al., 2000), the present study identified several amino acids outside of the copper-binding motif that appear to be critical for Cox17p function. In a subset of the *cox17* mutants, the specific loss of COX subunit II suggests that Cox17p may function to provide copper to the Cu_A site on subunit II, acting upstream of Sco1p. As with human COX17, many questions regarding Cox17p function remain to be answered, including mitochondrial

localization and uptake, physical interactions with other proteins, the number of bound copper ions, and the exact role of Cox17p in the assembly of COX.

While a genetic interaction has been shown between Cox17p and its proposed target protein, Sco1p, a physical interaction has never been demonstrated between the two proteins. In the absence of a physical interaction with a target protein, Cox17p remains classified as a "putative" copper chaperone. In an attempt to show an interaction between the Cox17p and Sco1p, I, and others, have used the two-hybrid assay. In all instances, the technique failed to demonstrate an interaction. There are several possible explanations for the lack of detectable interaction, including that the fusion proteins used in the assay abolish the interaction or that the fusion proteins do not become properly localized to the nucleus. In addition, if the association between Cox17p and Sco1p is extremely transient, the two-hybrid system may not be sensitive enough to detect the interaction. Coimmunoprecipitation experiments have also been used by a number of groups, including our lab, as a second method of identifying an association between Cox17p and Sco1p. As with the two-hybrid experiments, an interaction has not been shown by coimmunoprecipitation. While neither technique may be sensitive enough to detect an interaction, the negative result obtained using the two different techniques also could imply that the two proteins do not interact. Although the genetic interaction between Cox17p and Scop1 suggests that the two proteins function in the same pathway, the existence of an intermediate protein acting between Cox17p and Sco1p in the mitochondrial copper transport pathway cannot be excluded at this time.

All of the *cox17* mutants described in this thesis require further characterization to determine how the amino acid alterations affect the structure and function of the mutant

Cox17p. Conceivably, the introduced mutations could affect the stability of Cox17p in the cytosol or in the mitochondrial intermembrane space. In addition, amino acid substitutions may affect protein structure/function at any step in the mitochondrial copper transport pathway, including copper-binding, mitochondrial localization, mitochondrial uptake, docking with Sco1p, and transfer of copper to Sco1p. To address whether the mutant proteins are stable, pulse-chase experiments using metabolic labelling with ^{35}S-methionine could be carried out. An increase in the rate of mutant protein turnover relative to wild-type protein would indicate that the mutant protein is unstable.

It is currently unclear how many copper ions are bound to wild-type Cox17p. When purified from yeast, native Cox17p was found to bind two atoms of copper per molecule of protein (Beers et al., 1997). Cox17p purified as a fusion protein from *E.coli* coordinated two atoms of copper (Srinivasan et al., 1998). However, untagged Cox17p purified from *E. coli* bound three atoms of copper (Heaton et al., 2000). While it was argued that Cox17p purified as a fusion protein did not achieve its correct structure after cleavage of the tag and could not bind its full complement of copper (Heaton et al., 2000), it still remains to be determined whether expression of Cox17p in the heterologous environment of the bacterial cell leads to the correct metallation of the protein. The purification of untagged Cox17p in the yeast cell, followed by atomic absorption spectroscopy, may be useful in resolving the number of copper ions bound by Cox17p. To assess whether metal content is altered in Cox17p mutants, purified mutant protein could also be subjected to atomic absorption spectroscopy.

The mechanism by which Cox17p is localized and imported into the intermembrane space of the mitochondrion remains completely unknown. Currently, two

mitochondrial channels, porin and the general import/insertion pore of the TOM (translocase of the outer membrane) complex, have been identified (Antos et al., 2001). Porin, also known as the voltage-dependent anion-selective channel (VDAC), is the most abundant protein of the mitochondrial outer membrane and forms a pore that allows the passage of small molecules across the outer membrane (Krimmer et al., 2001). It is currently unclear if very small proteins, such as Cox17p, are able to cross the outer mitochondrial membrane via the porin channel. However, since a porin null mutant strain is viable on nonfermentable carbon sources, it is unlikely that Cox17p uses porin exclusively for membrane translocation. In light of the recent demonstration that apocytochrome c, a small intermembrane space protein, interacts with the TOM complex during mitochondrial uptake (Diekert et al., 2001), it would be worthwhile to investigate whether Cox17p associates with components of the TOM complex during mitochondrial import. Cross-linking experiments, designed to maintain any protein-protein interactions involving Cox17p, as well studies with TOM complex mutants, in which Cox17p would be retained in the cytosol if an interacting TOM complex component was not functional, would be two approaches for studying mitochondrial uptake mediated by members of the TOM complex. Cross-linking studies aimed at investigating Cox17p mitochondrial uptake may also be able to address whether copper is normally bound to Cox17p during passage across the outer membrane.

It has recently been reported that a small percentage of cellular SOD1 and its copper chaperone, CCSp, localize to the mitochondrial intermembrane space (Sturtz et al., 2001). In a *cox17* null mutant strain, mitochondrial SOD1 retains function, suggesting that Cox17p is not involved in the shuttling of copper to SOD1 in the

intermembrane space. Like Cox17p, neither CCSp nor SOD1 contain any previously identified N-terminal or internal mitochondrial targeting sequences. Although both CCSp and SOD1 are somewhat larger than Cox17p, with molecular weights of 27 and 32 kDa, respectively (Lamb et al., 1999), a common mechanism may be involved in the mitochondrial uptake of all three proteins. Studies of the mitochondrial import of CCSp and SOD1 may provide insight into the process of Cox17p outer membrane translocation.

While the mutagenesis study described in this thesis has identified a number of important amino acid residues, the general structure of Cox17p remains unknown. Because Cox17p does not share homology with any other known yeast protein, the structure of the protein cannot be predicted by computer modelling. Although a number of groups in the field have attempted to generate a detailed structure of Cox17p by X-Ray crystallographic techniques, no one has yet succeeded in obtaining Cox17p crystals. To determine the structure of Cox17p, nuclear magnetic resonance (NMR) may be a more useful approach. Unlike X-ray crystallography, NMR has the advantage of being carried out in solution and has been used to characterize proteins with disordered structures (Uversky, 2002).

Based on the high proportion of disordering and charged amino acid residues in Cox17p, I have suggested that Cox17p may be a natively unfolded protein. Although a substantial amount of biophysical characterization is needed to confirm this hypothesis, a number of experimental approaches are currently available to investigate the biophysical properties of a given protein (Denning et al., 2002). For example, determination of the Stokes radius of the protein can give some structural information. In a natively unfolded protein, the Stokes radius tends to be large relative to the molecular weight of the protein,

indicating that the protein does not adopt a compact structure. Sedimentation velocity experiments provide information regarding the mass, size and degree of compactness. Unexpectedly small sedimentation coefficients can be indicative of high drag forces, suggesting that a protein has a low degree of compactness. Spectral analyses, including circular dichroism (CD) and Fourier Transform Infrared (FTIR) spectroscopy, allow estimations of the α-helical, β-structure, and random-coil content of a protein. Intrinsically disordered proteins typically have a low content of ordered secondary structure. Proteinase K sensitivity assays are an additional way of assessing whether a protein is natively unfolded, since the highly flexible nature of intrinsically disordered proteins causes them to be more sensitive to the nonspecific protease.

In the case of a few *cox17* mutants, it appears that misassembled COX may be present. One technique, electron paramagnetic resonance (EPR) spectroscopy, allows the environments of Cu_A and heme a to be examined. In wild-type COX, characteristic signals are detected for Cu_A and heme a, while the Cu_B and heme a_3 centres are normally EPR-silent due to the spin coupling of the metals. When Cu_B is absent, a strong signal for heme a_3 is detected (Hiser et al., 2000). To determine if COX is misassembled in the mutants strains, EPR studies could be carried out on isolated COX. Using EPR, it should be possible to determine which copper centres and heme moieties are present, and whether the environments of the hemes are altered with respect to wild-type COX. It would be interesting, as well, to compare the EPR results to those of the misassembled *sco1* S240F mutant. These studies may be able to help determine if the inability or decreased ability to provide copper to COX subunit II affects the stability of the prosthetic groups on COX subunit I.

Although the order of COX subunit assembly has not been determined in yeast cells as of yet, two-dimensional polyacrylamide gel electrophoresis studies in cultured human cells have provided the basis for a model of the COX assembly pathway (Nijtmans et al., 1998). According to the model of Nijtmans *et al.*, the first species in the assembly pathway consists only of subunit I. The second species is comprised of subunits I and IV. With the exception of subunits VIa and VIIa or VIIb, all of the remaining subunits are incorporated into the third species, including the two remaining core subunits, COX II and COX III. The introduction of subunits VIa and VIIa or VIIb into the COX complex completes holoenzyme assembly. While this model gives a general idea of the order of subunit assembly, it is still unclear when the various COX prosthetic groups are incorporated. Because the two heme groups and the Cu_B site are located within the transmembrane region of COX I, it suggests that these prosthetic groups may be inserted as an early step, prior to the assembly of the majority of the subunits that interact with or surround COX I. Since it appears that COX II is assembled at a later stage than COX I and that the Cu_A site on COX subunit II is more exposed, it seems likely that copper is inserted at the Cu_A centre at a later timepoint than at the Cu_B site. The temporal separation of copper insertion at the two sites may indicate that copper incorporation occurs via two separate mechanisms, and may provide an explanation for the existence of two copper delivery pathways, namely the Cox11p pathway and Cox17p/Sco1p pathway (Figure 6-1). The idea that Cu_A insertion occurs at a later stage is supported by the subset of *cox17* mutants that display a specific loss of COX subunit II, with a much less significant decrease in COX subunit I. Whether the absence of

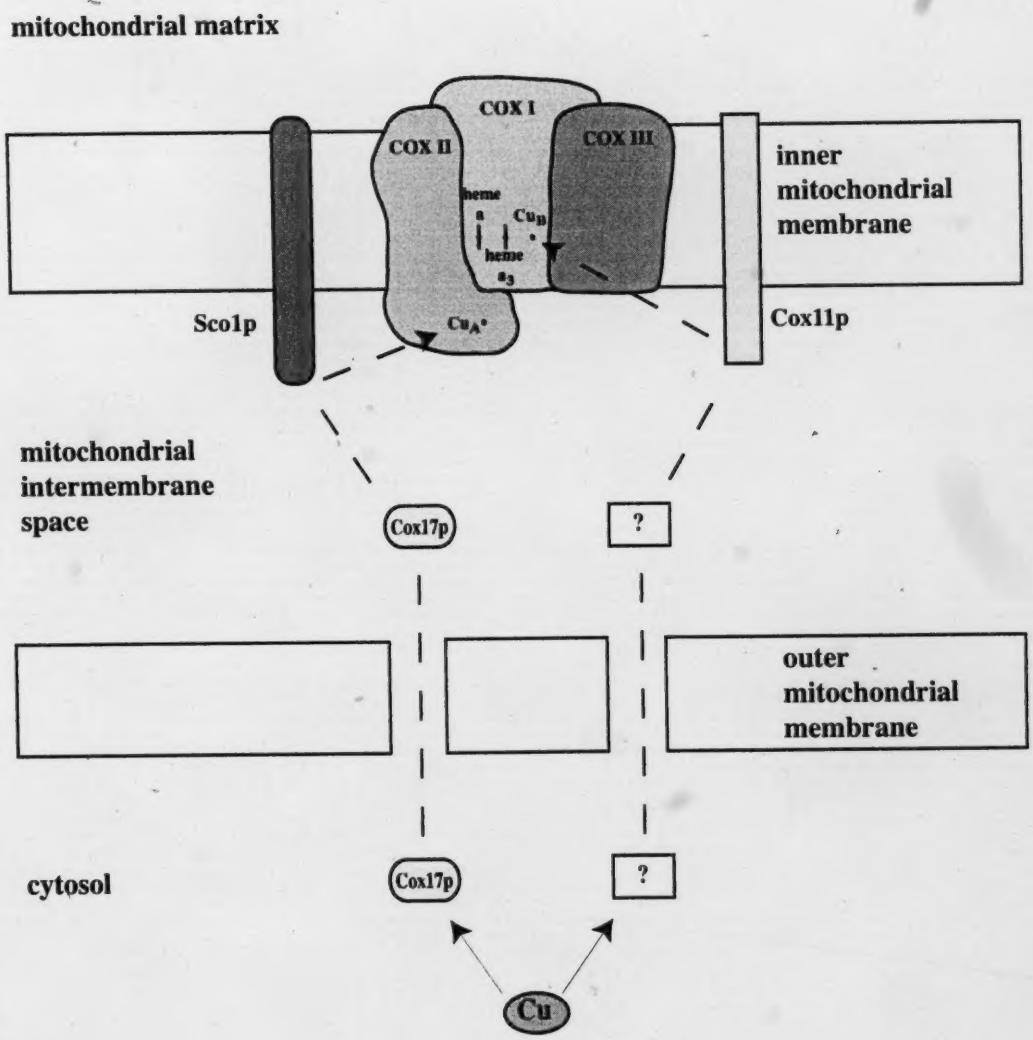

Figure 6-1. Model for the delivery of copper to cytochrome oxidase. Copper enters the cell by way of the high affinity copper transporter, Ctr1p. In the cytosol, copper is bound by Cox17p and transported across the outer mitochondrial membrane. In the intermembrane space, copper is passed from Cox17p to Sco1p. Sco1p, an inner mitochondrial membrane protein, facilitates the insertion of copper into the Cu_A on subunit II of cytochrome oxidase. In a second pathway, copper is shuttled from the cytosol to Cox11p, an inner mitochondrial membrane protein, by an unknown mechanism. Cox11p aids in the insertion of copper at the Cu_B site, located on subunit I of cytochrome oxidase.

copper at the Cu_A site renders subunit II unstable or prevents the next step in assembly from occurring remains to be determined.

The majority of the *cox17* mutants presented in this study also show somewhat decreased levels of COX subunit III, although the significance of this decrease is not currently understood. Since this *cox17* mutant study is the first report of COX III levels in COX assembly factor mutants, the examination of COX III levels in a number of different COX assembly factor mutant strains may provide a greater understanding of the relationship between COX II and COX III during COX assembly. Because it has been suggested that COX II and COX III are incorporated into the COX enzyme complex during the same step in the assembly pathway, a decrease or the absence of COX II may affect the efficiency of COX III insertion. A block in the assembly pathway at the stage of COX II incorporation may also leave COX III more susceptible to proteolytic degradation.

Conclusion

With the exception of complex II, all OXPHOS complexes are under the dual genetic control of the mitochondrial and nuclear genomes. From transcription of the nuclear subunit genes to the final assembly in the mitochondrial inner membrane, a multitude of gene products are devoted to the biogenesis of the OXPHOS complexes. While assembly factors presumably exist for all of these enzyme complexes, the proteins involved in COX biogenesis are the best characterized. Although considerable inroads have been made in deciphering the mechanism of COX assembly, the process remains poorly understood. The relevance of errors in COX biogenesis to human mitochondrial disease is becoming increasingly apparent, particularly considering that mutations in five

COX assembly factor genes have been implicated in human COX deficiency in the last five years. The mitochondrial copper transport pathway, which has been addressed in this thesis, represents only one small aspect of COX assembly. Given that over forty genes are required to produce functional COX, the assembly of the holoenzyme is clearly a complicated process. In addition to the unanswered questions regarding COX assembly in general, many issues relating to the mitochondrial copper transport pathway alone remain to be addressed. Some of these unresolved issues include whether additional assembly factors are involved, how copper insertion is achieved, how copper insertion fits into the overall scheme of COX assembly, and whether additional errors in mitochondrial copper transport have yet to be identified in human COX deficiency. To continue to unravel the process of COX biogenesis, much exciting work lies ahead.

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